The Circadian Clock Gene, Bmal1, Regulates Intestinal Stem Cell Signaling and Represses Tumor Initiation

Kyle Stokes, Malika Nunes, Chantelle Trombley, Danilo E. F. L. Flóres, Gang Wu, Zainab Taleb, Abedalrhman Alkhateeb, Suhrid Banskota, Chris Harris, Oliver P. Love, Waliul I. Khan, Luis Rueda, John B. Hogenesch, and Phillip Karpowicz

SUMMARY
The loss of circadian rhythms in the intestine leads to aberrant regulation of stem cell signaling pathways and increased tumor initiation.

BACKGROUND & AIMS: Circadian rhythms are daily physiological oscillations driven by the circadian clock: a 24-hour transcriptional timekeeper that regulates hormones, inflammation, and metabolism. Circadian rhythms are known to be important for health, but whether their loss contributes to colorectal cancer is not known. We tested the nonredundant clock gene Bmal1 in intestinal homeostasis and tumorigenesis, using the Apc\textsuperscript{min} model of colorectal cancer.

METHODS: Bmal1 mutant, epithelium-conditional Bmal1 mutant, and photoperiod (day/night cycle) disrupted mice bearing the Apc\textsuperscript{min} allele were assessed for tumorigenesis. Tumors and normal nontransformed tissue were characterized. Intestinal organoids were assessed for circadian transcription rhythms by RNA sequencing, and in vivo and organoid assays were used to test Bmal1-dependent proliferation and self-renewal.

RESULTS: Loss of Bmal1 or circadian photoperiod increases tumor initiation. In the intestinal epithelium the clock regulates transcripts involved in regeneration and intestinal stem cell signaling. Tumors have no self-autonomous clock function and only weak clock function in vivo. Apc\textsuperscript{min} clock-disrupted tumors show high Yes-associated protein 1 (Hippo signaling) activity but show low Wnt (Wingless and Int-1) activity. Intestinal organoid assays show that loss of Bmal1 increases self-renewal in a Yes-associated protein 1-dependent manner.


Keywords: Circadian Rhythms; Intestinal Stem Cells; Colorectal Cancer; Hippo Pathway.

Cancer is a disease that commences with the initiation of tumors, and progresses when hyperproliferative tumor cells develop into increasingly malignant phenotypes. How the body's physiology precisely...
Circadian rhythms are daily changes in physiology driven by the circadian clock: a self-sustaining, 24-hour, transcriptional feedback mechanism comprising the transcription factors Clock/Bmal1 and their repressors Per1-3/Cry1-2. In addition to this core mechanism, a regulatory loop composed of Nrl1d1-2 regulates the transcription of Bmal1 to confer robustness; post-transcriptional, translational, and epigenetic mechanisms also affect circadian clock function. Self-sustaining daily changes in the abundance and activity of the core clock components underlies 24-hour transcriptional rhythms that are present in nearly all cells of the body, where more than 40% of the genome is rhythmic. In doing so, the clock is thought to coordinate the timing of compatible and incompatible physiological processes throughout the body, thereby maximizing health and fitness. Daily circadian rhythms in hormones, inflammation, and metabolic processes are essential to health, and are the same physiological processes often dysregulated in tumor cells.

Circadian disruption, an outcome of frequent activities such as shift work, increases cancer incidence, and therefore a link between circadian rhythms and cancer has been actively sought. Cancers can harbor abnormal expression or mutations in circadian genes but the circadian rhythmicity of cancers has not been established. For instance, breast cancer cells have been shown to have normal or abnormal circadian gene expression, raising the question of whether certain cancers are clocked, or whether clock function is correlated with different stages of malignancy. Loss of circadian clock activity leads to cellular changes such as disruptions in metabolism, cell cycle, apoptosis, and the DNA damage response, cellular processes whose dysfunction are known to be protumorigenic. Animal models have shown that the loss of clock genes increases cancer, although it is worth noting that questions have been raised about the strains of mice used in these studies, as well as discrepancies in tumorigenesis noted between different clock mutants. In addition to the circadian clock as a transcriptional regulator, environmental factors that cause circadian disruption are complex and involve dysfunction in the orchestration of body-wide processes. Thus, the effect of circadian rhythms on colorectal cancer are poorly understood. How does the complete loss of circadian rhythms in the body, vs loss at the cellular level, lead to tumor initiation or progression? This is an important question, relevant in the role of circadian health as a factor in colorectal cancer prevention and treatment.

To test the role of the clock in colorectal tumorigenesis, we used the Apc\textsuperscript{min} mouse strain, which models human colorectal cancer in a mechanistically conserved manner. This strain has only 1 functional copy of the gene Apc: intestinal epithelial cells in these mice can undergo spontaneous loss of heterozygosity of the remaining allele and subsequent tumor initiation events at random positions in the epithelium. Apc mutant tumor-initiating cells are reminiscent of proliferative intestinal stem cells (ISCs), which divide throughout life to produce differentiated intestinal cells and regenerate intestinal epithelia. This is because the Apc gene is a negative regulator of Wnt signaling, a pathway required for the maintenance and proliferation of Lgr5+ (Leucine-rich repeat-containing G-protein coupled receptor 5) ISCs. Inflammation is also a critical factor in regeneration, and potent driver of tumorigenesis in the intestine. Recent studies have shown that intestinal cells can assume a fetal-like state during regeneration, and inflammation can activate the Hippo tumor-suppressor pathway to promote regeneration. Thus, both the Wnt and Hippo pathways are regenerative factors that are co-opted during tumorigenesis to drive tumor growth.

We previously showed that regeneration, in both Drosophila and mice, is regulated by the circadian clock. ISCs show 24-hour proliferation and cell signaling characteristic of circadian rhythmicity, and loss of clock function causes arrhythmic and dysfunctional regeneration. These same processes are regulated by the clock in epidermal stem cells, suggesting they are conserved in epithelia. We hypothesized that complete loss of circadian rhythms would increase intestinal tumorigenesis. Congenic Apc\textsuperscript{min} mice lacking Bmal1 have a 2-fold higher tumor initiation probability, which is recapitulated in Apc\textsuperscript{min} mice housed under constant light. In the intestinal epithelium, the circadian clock regulates a small number of rhythmic genes, including the Hippo signaling pathway transducer Tead4. However, Apc\textsuperscript{min} tumors show reduced circadian rhythms in vivo, and tumor cells have no capacity to generate cell-autonomous circadian rhythms in vitro, showing that loss of rhythms is linked to tumorigenesis. Furthermore, clock-dead Apc\textsuperscript{min} + Bmal1 mutant tumor organoids down-regulate the Wnt pathway and up-regulate the Hippo pathway, and show increased Hippo-dependent self-renewal. Our results show that the circadian clock is a physiological repressor of early tumor-initiation events, and that the core clock gene Bmal1 participates in the remodeling of stem cell-regenerative signaling pathways in the epithelium.

Results

Loss of Bmal1 Increases Tumor Initiation in the Intestine

Bmal1\textsuperscript{-/-} mutant mice are viable littermates from heterozygous crosses but show no circadian rhythms or cell activity. We crossed Apc\textsuperscript{min} mice on a C57BL/6 background with Bmal1\textsuperscript{-/-} null mutant mice also on a C57BL/6 background, then offspring were backcrossed 7 generations

Abbreviations used in this paper: ANOVA, analysis of variance; BH, Benjamini-Hochberg; cKO, conditional knockout; GSEA, Gene Set Enrichment Analysis; ISC, intestinal stem cell; Lgr5+, Leucine-rich repeat-containing G-protein coupled receptor 5; LL, constant light cycle; PBS, phosphate-buffered saline; PHH3, phosphorylated histone H-3; Tnf, tumor necrosis factor; Wnt, Wingless and Int-1; Yap, Yes-associated protein 1.

© 2021 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
to create a congenic strain. Littermates from congenic heterozygous Apc\textsuperscript{min}/+; Bmal1\textsuperscript{+/+} crosses were examined at 3 months of age to avoid age-related phenotypes.\textsuperscript{23} Bmal1-deficient mice have low and arrhythmic serum levels of corticosterone, a known regulator of circadian physiology.\textsuperscript{52} We performed the same experiment using Apc\textsuperscript{min} control organoids (Apc\textsuperscript{min}/+; Bmal1\textsuperscript{+/+}) and Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} organoids (Apc\textsuperscript{min}/+; Bmal1\textsuperscript{-/-}) derived from microdissected tumors (Figure 4A). Apc\textsuperscript{min} organoids express 238 rhythmic genes and Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} express 263, using the same criteria as described earlier (Figure 4B, Supplementary Table 1). The circadian clock was dysfunctional in both genotypes. Even control tumors with wild-type Bmal1 do not have self-sustaining circadian clock transcription, indeed these down-regulate most clock components (Supplementary Table 2). Only 4 rhythmic genes overlapped between genotypes, showing that the 24-hour variation in gene expression is unique (Figure 4C). The top genes in Apc\textsuperscript{min} (Golga1, Dlk1, Rnf128) or Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} (Mlxipl, Msno1, Fam76b) were tested over 2 days, and although some showed variation over 24-hour organoid culture (Figure 4D), these were not self-sustaining circadian rhythms over 48 hours, suggesting stochastic expression, with the exception of Rnf128 and Msno1, which have 24-hour rhythms (Figure 4E). We conclude that tumor organoids show 24-hour variation in gene expression, independent of Bmal1, but have no autonomous, self-sustaining, rhythmic, transcriptional clock mechanism present.

The Normal Epithelium Shows Self-Sustaining Circadian Clock Function, but Tumors Do Not

To test how Bmal1 functions as a transcriptional regulator in the epithelial crypt, we derived organoids from congenic wild-type control mice (Apc\textsuperscript{+/+}; Bmal1\textsuperscript{+/+}) vs Bmal1\textsuperscript{-/-} mice (Apc\textsuperscript{+/+}; Bmal1\textsuperscript{-/-}). Twelve hours after passaging, organoids were synchronized using a single pulse of the glucocorticoid dexamethasone, and then fractions were collected every 2 hours from 24 to 48 hours after synchronization to avoid immediate early response gene effects.\textsuperscript{56} RNA was sequenced, and transcriptomes were analyzed by MetaCycle.\textsuperscript{57} (Figure 3A). Comparing wild-type vs Bmal1\textsuperscript{-/-} organoid circadian expression showed that only 41 genes are expressed rhythmically in a Bmal1-dependent manner (base expression, >0.5; relative amplitude, >0.05; BH (Benjamini-Hochberg), q < 0.1 (Figure 3B, Supplementary Table 1). These include well-known circadian clock components such as Bmal1 itself, Nr1d1-2, Nr1f3, Per3, Cry1-2, and Tef (Figure 3C, Supplementary Table 1). Additional rhythmic targets, previously implicated in ISC biology, are notable including the markers: Anxa2, 7, 8\textsuperscript{β}, differentiation regulators: Bmp3, Cav2, Ephb3;\textsuperscript{58} growth regulators: Nras;\textsuperscript{59} DNA repair component: Parp3;\textsuperscript{60} and regeneration regulators: Kit and Tead4.\textsuperscript{55,59} (Figure 3C, Supplementary Table 1). No cell-cycle regulators were detected. A second organoid assay, performed over 2 days to test a subset of the top rhythmic genes, showed that circadian clock genes showed consistent self-sustaining 24-hour rhythms (Figure 3D). However, the Hippo pathway component Tead4 showed time-dependent variation that had less consistent 24-hour rhythmicity. These results suggest that Bmal1 regulates clock transcription in the normal intestinal epithelium, but that its targets are limited to a small number of genes that include several known ISC regulators.

We measured proliferation in intestinal crypts and tumors using Ki67, a marker of cell-cycle activity, and phosphorylated-histone H3 (PHH3), a marker of mitosis. Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} intestinal crypts and tumors show stronger expression levels of Ki67 and an increased number of Ki67+ cells (Figures 1E and F and 2A and B). PHH3 cell number is increased in Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} crypts and tumors and shows daily variation (Figures 1G and H and 2E and F). This indicates that loss of Bmal1 increases proliferation in the Apc\textsuperscript{min} background. Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} tissues also showed reduced cell death (Figures 1I and 2G), as marked by cleaved caspase-3. Histopathologic analysis showed that Bmal1 loss slightly reduces dysplasia (Figure 1J), but does not affect tumor size (Figure 1K). To test whether tumor growth is increased in an age-dependent fashion, we examined mice at earlier stages of tumor initiation. One-month-old mice lacking Bmal1 did not show an increase in tumor number yet (Figure 1L and M), but Ki67 expression levels and/or numbers were increased in tumors (Figure 1N and O) and in crypts (Figure 2C and D). Thus, the phenotype of Bmal1 loss of function is the generation of more tumors through increased proliferation. We conclude that in this manner Bmal1 negatively regulates tumorigenesis.

A recent study proposed that the circadian clock regulates the Wnt pathway ligand, Wnt3A, in the Paneth cells of intestinal organoids.\textsuperscript{24} Because dysfunction of Wnt is a driver of tumorigenesis, we tested if increased Wnt signaling disruption precedes the increase in Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} tumor initiation. β-catenin, the transducer of Wnt signaling, was unaltered (Figure 2H and I). Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} untransformed crypts showed reductions in the number (Figure 2J) and size (Figure 2K) of crypts, but an increase in the overall number of crypt cells present (Figure 2L). These changes did not resemble the heightened proliferation that would be expected from Wnt increased activity. However, we found an increase in the production of secretory cell types, including Paneth cells (lysozyme-positive) and goblet cells (Alcian Blue) in the Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} intestine (Figure 2M–P). These data are consistent with previous reports showing Hippo pathway effects on secretory cell differentiation.\textsuperscript{43,55}
A

B

C # of Tumors >1mm

D # of Tumors <1mm

E

F

G

H

I

J

K

L

M

N

O

1 month old mice

web 4C/FPO

4 Stokes et al Cellular and Molecular Gastroenterology and Hepatology Vol. ■, No. ■
Epithelial Self-Renewal Is Regulated by Bmal1

We next compared changes in gene expression between genotypes over the full 24-hour period. There were 111 genes changed, either up-regulated or down-regulated, when comparing Bmal1+/+ controls and Bmal1−/− mutant organoids (Supplementary Table 2). Bmal1−/− organoids showed 67 genes increased in expression (Figure 5A, Supplementary Table 2), which included inflammatory markers such as Reg3a, Reg3b, and Cntf, indicative of a stress/inflammatory state.60 Compared with non-transformed wild-type controls, Apcmin−/− tumor organoids up-regulate genes involved in proliferation, Wnt signaling, and inflammation, but down-regulate genes involved in metabolism and clock activity (Supplementary Tables 2 and 3). This is consistent with the role of Apc as a Wnt pathway repressor. Apcmin−/+ + Bmal1−/− organoids up-regulate or down-regulate 2398 genes when compared with Apcmin−/− organoids (Supplementary Table 2). Of note, Wnt target genes and apoptosis genes were down-regulated in the absence of Bmal1, but Hippo target genes, as well as many genes involved in proliferation, were up-regulated (Figure 5B and C, Supplementary Tables 2 and 3). To confirm the increase in Hippo signaling, we performed Gene Set Enrichment Analysis (GSEA)61 using a gene set containing genes previously found to be regulated by the Hippo pathway target genes, as well as many Hippo target genes, as well as many genes involved in proliferation, were up-regulated (Figure 5B and C, Supplementary Tables 2 and 3). To confirm the increase in Hippo signaling, we performed Gene Set Enrichment Analysis (GSEA)61 using a gene set containing genes previously found to be regulated by the Hippo pathway target Axin2 (not shown). Treatment of Apcmin−/−; Bmal1−/− organoids with the Yap inhibitor abolished their increased self-renewal (Figure 5MD, and treatment with a Tead4-specific inhibitor showed a similar effect (not shown). Overall, these results suggest that Bmal1 suppresses a Hippo-dependent, self-renewal pathway in the intestinal epithelium, and in its absence the self-renewal of ISCs or tumor-initiating clones is increased.

Epithelial Bmal1 Contributes to Increased Tumor Initiation

To what extent does the epithelium play a role in the increased tumor initiation of Bmal1−/− mutants in vivo? To address this question, we crossed Apcmin−/− mice with Villin-Cre67 and Bmal1fllox/fllox mice68 to delete exon 8—the DNA transactivation domain—thereby conditionally abolishing Bmal1 and thus circadian transcriptional activity only in the epithelial cells in the Apcmin−/− background (Figure 6A). Three-month-old Apcmin−/− conditional knockout (cKO) control mice (Apcmin−/−; Bmal1fllox/fllox) and Apcmin−/+ + Bmal1−/− cKO mice (Apcmin−/−; Vill−/−; Bmal1fllox/fllox) were tested, showing that the Apcmin−/+ + Bmal1 cKO mice had an equal number of large tumors, but 2-fold higher small tumors (Figure 6B and C). This shows that loss of Bmal1 in the epithelium partially recapitulates the full mutant phenotype.
Figure 2. Loss of Bmal1 alters tissue homeostasis. (A and B) Ki67+ expression was more intense and increased Ki67+ cells were present in Apcmin; Bmal1+/+ crypts at 3 months of age (t test, \(P = .0012\)). Scale bar: 50 μm. (C and D) Earlier, at 1 month, these proliferation differences also were present, suggesting the tissue of Bmal1−/− mutants is primed for increased tumor initiation before tumors double in number (t test, \(P = .0166\)). Scale bar: 50 μm. (E and F) Slightly increased mitosis (PHH3+) was observed in Apcmin; Bmal1 mutant crypts at 3 months (ANOVA \(f_{2, 218} = 3.386; P = .0352\)). Scale bar: 25 μm. (G) Reduced apoptosis (caspase 3 [Cas3]−) was observed in Apcmin; Bmal1−/− crypts (t test, \(P = .031\)). (H and I) β-catenin positive (activated) nuclei were equal, suggesting that Wnt signaling was equally active in Apcmin control (left) and Apcmin; Bmal1 mutant (right) crypts. Scale bar: 50 μm. (J) Slightly fewer crypts were noted in Apcmin; Bmal1 mutant animals (t test, \(P = .0013\)). (K) Slightly reduced crypt depth was noted in the Apcmin; Bmal1 mutant (t test, \(P < .0001\)). (L) Slightly more cells also were found in the same (t test, \(P = .0123\)). (M and N) The number of secretory goblet cells (Alcian Blue) was higher in the Apcmin; Bmal1 mutant animals as well (t test, \(P < .0001\)). Scale bar: 100 μm. (O and P) Significantly more Paneth cells (lysozyme [Lyz]−) were present in Apcmin; Bmal1−/− mutant animals (t test, \(P = .0005\)). Scale bar: 25 μm. Histologic quantification is shown for \(n = 10\) crypts/regions per animal, and \(n \geq 3\) animals per condition. All error bars represent SEM. β-Cat, β-catenin; DAPI, 4',6-diamidino-2-phenyindole.
Are there other factors that drive tumorigenesis in the Bmal1−/− mutant? The circadian clock regulates many different rhythms in physiology, including daily cycles of metabolism and the endocrine and immune systems.4,12 Inflammation is an important driving factor in colorectal tumorigenesis,39 and the circadian clock also recently was shown to regulate inflammation in the intestine.69,70 We therefore asked whether Apcmin + Bmal1−/− mice have...
Figure 4. Analysis of circadian transcription in the tumor epithelium. (A) Tumor organoids were derived from Apc\textsuperscript{min} and Apc\textsuperscript{min} + Bmal1\textsuperscript{+/−} mice, and then RNA was analyzed by sequencing as shown in Figure 2 (n = 2 organoid lines/genotype). (B) Heat maps median-normalized gene expression (Exp./Med.) ordered by MetaCycle phase show all rhythmic genes. (C) Venn diagram showing that there is nearly no overlap between rhythmic transcripts identified from wild-type and mutant samples. (D) Graphs of the RNA sequencing of the top 3 genes from each genotype: Golga1 (q = 0.015), Dolk (q = 0.015), Rnf128 (q = 0.015), Mixipl (q = 0.0053), Msmo1 (q = 0.0053), and Fam76b (q = 0.0055). (E) Validation test using 2-day quantitative polymerase chain reaction (qPCR) indicated that only Rnf128 (ANOVA, $f_{(12, 24)} = 1.378, P = .2427$) and Msmo1 (ANOVA $f_{(12, 26)} = 4.613, P = .0005$) show self-sustaining rhythms. All error bars represent SEM. Dex, Dexamethasone; RPKM, Reads Per Kilobase of transcript, per Million mapped reads.
Circadian Disruption Increases Tumorigenesis

A. Circadian Clock Gene Expression

B. Wnt Signaling and Hippo Signaling

C. Downregulated Genes in Apc\textsuperscript{min}; Bmal1\textsuperscript{-/-} vs. Apc\textsuperscript{min}; Bmal1\textsuperscript{+/+}

D. Genes Upregulated by Yap in Apc\textsuperscript{min}; Bmal1\textsuperscript{+/+}

E. Single Cell Dissociation

F. Immunohistochemistry

G. # of Clones

H. # of Crypts

I. Inhibitor

J. Single Cell Dissociation

K. Expression by qPCR

L. Ereg

M. # of Clones

---

FLA 5.6.0 DTD  JCMGH863 proof  14 October 2021  12:35 pm  cc DVC
increased inflammation, which together with the epithelium could contribute to tumor initiation. Blood was analyzed from the full-body KO and cKO mice, and tested for proinflammatory cytokines. Analysis of a panel of cytokines showed that $Apc^{min} + Bmal1^{−/+}$ mice have increased levels of Tumor Necrosis Factor (TNF) (Figure 6D), a clock-regulated proinflammatory cytokine.46 However, $Apc^{min} + Bmal1$ cKO mice did not show this increase in TNF. To test whether local inflammation surrounding the epithelium is higher, CD45+ blood cell infiltration into tumors was quantified. cKO controls, $Apc^{min} + Bmal1$ cKO, and $Apc^{min} + Bmal1^{+/−}$ tumors, have equivalent lower numbers of CD45+ cells, while $Apc^{min} + Bmal1^{−/+}$ tumors have significantly higher CD45+ numbers, indicating a proinflammatory state (Figure 6E and F). We conclude that global circadian disruption in $Apc^{min} + Bmal1$ cKO mice drives a protumorigenic inflammatory phenotype that is not present in the $Apc^{min} + Bmal1$ cKO.

Yap Activity Is Increased in Bmal1 Mutant Tumors

The Hippo signaling transducer, Yap, is required for tumorigenesis in the $Apc^{min}$ colorectal cancer model.43 We therefore probed $Apc^{min}$ controls and $Apc^{min} + Bmal1^{−/+}$ with an antibody marking Yap to determine if Hippo signaling is perturbed when $Bmal1$ is absent. $Apc^{min} + Bmal1^{−/+}$ epithelial tumors have higher levels of Yap, and the signal shows an active/nuclear localization compared with $Apc^{min}$ controls (Figure 7A and B). Protein was extracted from the tumors of $Apc^{min}$ control and $Apc^{min} + Bmal1^{−/+}$ mice and analyzed by Western blot to further test Hippo activity. Both total Yap and activated Yap (nonphosphorylated S127) are higher in $Apc^{min} + Bmal1^{−/+}$ tumors (Figure 7C). This shows that Yap expression and activity is higher in $Bmal1$ mutant tumors consistent with the Yap signature of $Apc^{min} + Bmal1$ tumor organoids (Figure 5D).

To address whether this Yap increase is owing to epithelial $Bmal1$ function, $Apc^{min}$ cKO control mice and $Apc^{min} + Bmal1$ cKO mice were tested for markers of the Wnt and Hippo pathways. $Apc^{min} + Bmal1$ cKO mice have increased levels of Yap localized in the nucleus (Figure 7D and E) similar to those in $Apc^{min} + Bmal1^{−/+}$ mutants. Expression of the Hippo pathway component $Tead4^{−/−}$ also was increased in $Apc^{min} + Bmal1$ cKO tumors (Figure 7F and G). We further probed for the Wnt target, $Axin2$, and the Yap target, $Ereg$, in the $Apc^{min} + Bmal1$ cKO tumors. The $Bmal1$ cKO tumors have lower Wnt but higher Hippo pathway activity (Figure 7H and I). The results of the $Axin2$/$Ereg$ analysis mirror those of the $Lgr5$/$Tead4$ expression almost exactly, both indicating that the $Bmal1$ tumors have higher Hippo signaling at the expense of Wnt. Of note, Wnt markers ($Lgr5$ and $Axin2$) and Hippo markers ($Tead4$ and $Ereg$) are enriched in separate cells, suggesting these reflect changes in the tumor cell population. Together, these data show that $Bmal1$ cKO tumors have lower Wnt but higher Hippo activity, consistent with the $Bmal1$-dependent transcriptional program in $Apc^{min} + Bmal1^{−/+}$ organoids (Figure 4B–D). We conclude that $Bmal1$ negatively regulates the Hippo pathway in the tumor epithelium and, in its absence, these display higher Hippo activity.

In Vivo Tumors Have Weak Circadian Rhythms

Because tumor organoids do not show rhythmic circadian clock transcription in vitro (Figure 4), we asked whether circadian clock disruption is a general feature of intestinal tumors. We re-examined $Apc^{min}$ control mice, housed under normal photoperiod, to determine whether heterogeneity in clock activity or Wnt/Hippo signaling is present in the different tumors initiated. Individual tumors from a cohort of mice over a 24-hour timeline were microdissected ($n = 30$ mice, 5 per time point), and compared with adjacent nontransformed tissue for gene expression ($n = 180$ tumor/tissue pairs, $n = 30$ per time point). $Apc^{min}$ tumors showed lower expression of the clock genes $Nr1d1$, $Bmal1$, and $Per2$ than the surrounding

**Figure 5. (See previous page).** Epithelial self-renewal is regulated by $Bmal1$. (A) Heat map showing 24-hour fold change in relative mean expression of $Bmal1^{−/+}$ organoids compared with control organoids. Circadian clock genes were down-regulated, and inflammation markers were up-regulated in $Bmal1$ mutant organoids compared with controls. (B) Heat map showing 24-hour fold change in relative mean expression of $Apc^{min} + Bmal1^{−/+}$ organoids compared with $Apc^{min}$ control organoids. Wnt pathway targets were down-regulated, whereas Hippo pathway targets were up-regulated. Cell proliferation genes also showed an increase in $Apc^{min} + Bmal1^{−/+}$. (C) GO (Gene Ontology) analysis of pathways down-regulated by $Apc^{min} + Bmal1$ mutant tumor organoids, relative to $Apc^{min}$ controls. Wnt signaling was decreased, whereas cell proliferation was increased in $Bmal1$ mutants, together with changes in transcriptional regulation. A Benjamini cut-off value of 0.05 was used for GO analysis. (D) $Apc^{min} + Bmal1^{−/+}$ have up-regulated Yap signaling compared with $Apc^{min}$ controls (enrichment score, 0.44; normalized enrichment score, 2.083), nominal $P$ value = .000, False Discovery Rate (FDR) $q$ value = 0.000, Family Wise Error Rate (FWER) $P$ value = .000. (E) After derivation, individual organoids from each genotype were dissociated into single cells and plated separately to assess the number of subclones, and the number of crypt buds per subclone. (F) Representative images of organoid subclones. Scale bar: 100 μm. (G) Individual $Bmal1^{−/+}$ organoids developed more subclones ($t$ test, $P = .0004$), indicating increased self-renewal ability. (H) $Bmal1^{−/+}$ subclones had more crypt buds, indicating further increased self-renewal of SCs ($t$ test, $P < .0075$). (I) Schematic shows clonal analysis of $Apc^{min}$ organoids. (J) Representative images of $Apc^{min}$ control and $Apc^{min} + Bmal1^{−/+}$ mutant organoids were treated with Yap inhibitor; dimethyl sulfoxide (DMSO) is the vehicle control. $Apc^{min} + Bmal1^{−/+}$ organoids showed increased expression of the Yap target gene $Ereg$, which was reduced by Yap inhibitor (ANOVA, $f_{(3,10)} = 19.55; P = .002$). (M) Organoids were pretreated with Yap inhibitor before subcloning. $Apc^{min} + Bmal1^{−/+}$ organoids showed increased self-renewal, which was disrupted by pretreatment of Yap inhibitor (ANOVA, $f_{(3,24)} = 7.087; P = .00144$). Data show $n = 10$ clones from 2 to 3 lines for quantifications. All error bars represent SEM. Inflamm, Inflammation; qPCR, quantitative polymerase chain reaction; Red-Ox, Reduction-Oxidation; Rel Mean Exp, Relative Mean Expression; Sig, Signalling; Yap-i, Yap-inhibitor.
nontransformed tissue, and at the population level (average) clearly showed reduced circadian rhythms in vivo (Figure 8A–F). This expression was heterogeneous but nearly all individual tumors showed lower clock gene expression than the surrounding nontransformed tissue. We further tested circadian clock activity in the epithelium by staining for the clock component, \( \text{Nr1d1} \),\(^2\) in \( \text{Apc}^{\text{min}} \) controls and \( \text{Apc}^{\text{min}} + \text{Bmal1}^{-/-} \) mutants. \( \text{Nr1d1} \) showed daily changes in its levels and nuclear localization in the normal crypt epithelium of \( \text{Apc}^{\text{min}} \) control animals (Figure 8G and H). \( \text{Apc}^{\text{min}} + \text{Bmal1}^{-/-} \) crypts did not show these daily fluctuations but high \( \text{Nr1d1} \) levels at all times examined. In tumors present in the same animals tested, \( \text{Nr1d1} \) levels fluctuated in tumors in \( \text{Apc}^{\text{min}} \) controls at the same time as nontransformed tissue, and \( \text{Apc}^{\text{min}} + \text{Bmal1}^{-/-} \) had high levels at all times (Figure 8I and J). \( \text{Nr1d1} \) protein in tumors thus showed diurnal rhythms such as the surrounding tissue, corroborating the RNA analysis. Overall, we conclude that weak diurnal rhythms in clock components persist in tumors in vivo.

A circadian regulation of the Wnt pathway was reported previously in organoids,\(^{54}\) hence we examined Wnt signaling in these same samples. In vivo, the intestine showed daily changes in the Wnt pathway targets \( \text{Axin2} \),

---

**Figure 6. Epithelial \( \text{Bmal1} \) contributes to increased tumor initiation.** (A) \( \text{Bmal1} \) was expressed in the intestine of controls, but was absent in the intestine of \( \text{Bmal1} \) cKO (\( \text{Apc}^{\text{min}} \), \( \text{VilCre}^{+/+} \), \( \text{Bmal1}^{+/+} \)). In the liver, both controls and cKO expressed \( \text{Bmal1} \), indicating the specific deletion of \( \text{Bmal1} \) from the intestine (\( f_{\text{deg}} = 11.48; P = .0026; n = 3 \) per genotype). (B and C) \( \text{Apc}^{\text{min}} + \text{Bmal1} \) cKO (\( n = 7 \)) have an equivalent number of tumors greater than 1 mm, but significantly more tumors smaller than 1 mm, compared with \( \text{Apc}^{\text{min}} \) cKO controls (\( n = 8 \)) (t test, \( P = .027 \)). (D) \( \text{Tnf} \) is higher in \( \text{Apc}^{\text{min}} + \text{Bmal1}^{-/-} \) mutants compared with controls and both of the cKO genotypes (ANOVA, \( f_{\text{deg}} = 8.42 ; P = .0014; n = 4-6 \) mice per genotype). (E and F) Tumor tissue from \( \text{Apc}^{\text{min}} + \text{Bmal1}^{-/-} \) tumors show an increased number of infiltrating \( \text{CD45}^{+} \) blood cells than the other genotypes (ANOVA, \( f_{\text{deg}} = 6.824; P = .005 \)). Histologic quantification shown for \( n = 10 \) regions per animal, and \( n \geq 3 \) animals per condition. Scale bar: 10 \( \mu \)m. All error bars represent SEM. DAPI, 4',6-diamidino-2-phenylindole; \( \text{qPCR} \), quantitative polymerase chain reaction; Zeitgeber Time.
Figure 7. Yap activity is increased in Bmal1 mutant tumors. (A and B) Representative images showing increased Yap+ nuclear signal in the cells of Apc\textsuperscript{min} + Bmal1\textsuperscript{-/-} tumors (arrowheads show the cell nucleus in inset image). Yap-activated nuclei are present in higher numbers (t test, \(P = .0186\)). Scale bar: 50 \(\mu\)m. (C) Western blots showing increased levels of total and activated Yap in Apc\textsuperscript{min}; Bmal1\textsuperscript{-/-} mutant tumors. (D and E) Representative images showing increased Yap+ nuclear signal in the cells of Apc\textsuperscript{min} + Bmal1 cKO that are present in higher numbers (t test, \(P = .0009\)). Scale bar: 50 \(\mu\)m. (F and G) Control tumors show higher expression of Lgr5 RNA (blue) while Apc\textsuperscript{min} + Bmal1 cKO tumors show higher Tead4 RNA expression (red). The area of Tead4+ cells is significantly higher in Apc\textsuperscript{min} + Bmal1 cKO (ANOVA \(f_{(3, 36)} = 9.241; P = .0001\)). Scale bar: 50 \(\mu\)m. (H and I) Apc\textsuperscript{min} + Bmal1 cKO tumors show higher levels of the Yap target gene Ereg (blue), but lower levels of the Wnt target gene, Axin2 (red), relative to controls. The area of Ereg+ cells is significantly higher (ANOVA \(f_{(3, 36)} = 9.422; P < .0001\)). Histologic quantification shown for \(n = 5\)–10 regions per animal, and \(n \geq 3\) animals per condition. Scale bar: 100 \(\mu\)m. All error bars represent SEM.
Figure 8. In vivo tumors have weak circadian rhythms. (A and B) Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) graphs show transcript levels obtained from Apc\textsuperscript{min} mice housed under a light dark cycle. Individual tumors were compared directly with their adjacent nontransformed tissue taken from the ileum of the small intestine. Six time points over 24 hours were tested, from 5 mice per time point (n = 30 tumors per time point). Upper graphs show individual tumor expression, and lower graphs show the average of that time point. Nr1d1 expression in individual tumors is lower but rhythmic (2-way ANOVA, $f(5, 168) = 12.01; P < .0001$). (C and D) Bmal1 (2-way ANOVA, $f(5, 168) = 6.49; P < .0001$) also is lower but rhythmic. (E and F) Per2 shows lower expression in tumors relative to tissue as well ($f(1, 168) = 25.50; P < .0001$). (G and H) Nr1d1 antibody in nontransformed intestinal crypts tested at 3 time points over 24 hours (Zeitgeber Time [ZT]4, 12, 20). White outlines shows the crypt border. Apc\textsuperscript{min} controls have diurnal changes in Nr1d1 in crypt cells, while Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} nontransformed crypts show arrhythmic levels (2-way ANOVA, $f(2, 116) = 351.9; P < .0001$). (I and J) Nr1d1 staining in tumors from the same animals. Apc\textsuperscript{min} control tumors show diurnal changes, while Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} tumors are again arrhythmic (2-way ANOVA, $f(2, 27) = 127.4; P < .0001$), n = 3 mice per time point. Scale bar: 25 μm. Histologic quantification shown for n = 10 crypts/regions and n ≥ 3 animals per condition. All error bars represent SEM. DAPI, 4',6-diamidino-2-phenylindole.
Lgr5, Ccnd1, and cMyc (Figure 9A-H). The Hippo pathway genes Ereg and Tead4 similarly showed diurnal changes (Figure 9I-L). Although tumors showed heterogeneity in the expression of Wnt/Hippo target genes, at the population level daily changes were evident, with tumors generally showing increased levels of these cancer and proliferation-related pathways. To further analyze these data, we tested the Pearson correlations of clock genes to Wnt genes in all of these samples. These showed that the normal correlations between the timing of clock and Wnt activity were altered in tumors (Figure 9M and N). For instance, the negative correlation between Bmal1 and Per2 broke down, whereas...
correlations between Nr1d1 and Axin2, cMyc, or Ccnd1 increased. Wnt pathway genes were highest toward the end of the light phase (Zeitgeber Time 8–12), and these rhythms were exacerbated in tumors despite their lower clock activity. These data indicate that individual tumors are heterogeneous but all of them had reduced diurnal patterns of circadian clock components that did not correlate to reductions in protumorigenic signaling.

Circadian Rhythm Loss Potentiates Tumor Initiation

Circadian rhythms in mice are entrained by photoperiod, and the behavioral and physiological rhythms that ensue consolidate circadian clock function in tissues throughout the body.71 Our results thus far show the effects of Bmal1 loss in tumor initiation; however, it is not clear if this is a feature of disrupted circadian rhythms or noncircadian Bmal1 functions. To address this, we exposed Apcmin mice to constant light levels (LLs) that lead to circadian disruption12 and quantified tumor incidence at 3 months of age. Wild-type mice did not develop tumors in constant light, but Apcmin mice showed a 2-fold increase in both small (<1 mm) and large (>1 mm) tumor initiation in constant light compared with those under normal photoperiod (light dark cycle) (Figure 10A–C), essentially recapitulating the effects observed in the Apcmin + Bmal1 mutant (Figure 1C and D). This confirms that disruption of circadian rhythms itself was able to increase tumor initiation in the Apcmin background. We further tested Hippo signaling and cell proliferation in the LL circadian-disrupted tumors to see whether the same Bmal1-dependent increases were present. Apcmin mice exposed to constant light showed an increase in Yap-positive tumor cells, with concentrated staining in the nuclei (Figure 10D and E) identical to both the full body and conditional Bmal1 knockout mice (Figure 7A, B, D, and E). Ki67 was increased in the crypts (Figure 10F and G) and tumors of Apcmin LL mice (Figure 10H and I), similar to the Bmal1−/− mutant (Figure 1E and F). These data show that environmental disruption to circadian entrainment through increased light exposure leads to a Yap-active tumor phenotype that resembles the Bmal1 loss of function phenotype.

Discussion

The effect of circadian rhythms on tumorigenesis is a long-standing question. Studies have shown that shift workers have a higher cancer incidence,7,17,71 suggesting that circadian rhythm disruption is a risk factor. However, the epidemiologic data are not yet conclusive,14 and studies using animal models to test how clock genes relate to cancer also have been equivocal.13 Early reports have indicated that lymphoma was increased in Per2 mutant mice,28 and this same strain recently was shown to have increased liver and lung tumors.30,31 Indeed, Per2 mutant mice have increased intestinal tumorigenesis when crossed to the Apcmin model.29 However, Per2 is redundant with its homologs Per1 and Per3, and the circadian clock is active in the Per2 mutant, which has circadian rhythms present.2,73 Hence, it is not clear if the increased tumorigenesis resulting from Per2 loss is owing to its role as a circadian clock gene, or to its nonclock functions.

Using the Apc model of colorectal cancer we show that tumor initiation is regulated negatively by the circadian clock. Loss of the nonredundant clock gene, Bmal1, in a congenic Apcmin mouse strain, and circadian disruption in the Apcmin strain effected by constant light, both increased tumor number (Figures 1B–D and 10A–C). Conditional deletion of Bmal1 in the intestine also increased tumor number (Figure 6B and C), showing that Bmal1 and/or the epithelial clock specifically played a role in suppressing tumor initiation. These experiments confirmed that circadian rhythm disruption in the epithelium is a causal factor in tumorigenesis. Both Bmal1−/− organoids and Apcmin + Bmal1−/− organoids have increased self-renewal (Figure 5E–K), consistent with the idea that these have an enhanced ability to initiate tumors. Although proliferation was increased, we did not observe that Bmal1 loss increased tumor size (Figure 1K), suggesting that the suppressive effects of Bmal1 may be more complex than simply driving general tumor growth. These data provide insight into the precise stages of tumorigenesis, in which the circadian clock exerts its effects in this tissue.

Bmal1 loss decreases tumorigenesis in the skin,49 as does its binding partner Clock,74 whereas its negative regulator Per2 has the opposite effect in the skin and

Figure 9. (See previous page). Analysis of circadian clock, Wnt and hippo genes in the intestine. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) graphs from same samples as shown in Figure 8. The Wnt target genes were as follows: (A and B) Lgr5 (2-way ANOVA, f5, 168 = 2.50; P = .033), (C and D) Axin2 (2-way ANOVA, f5, 168 = 9.24; P = .0027), (E and F) Ccnd1 (1-way ANOVA, f1, 168 = 8.94; P = .0032), and (G and H) cMyc (2-way ANOVA, f1, 168 = 71.98; P < .0001), and were higher in tumors but also had diurnal changes similar to the surrounding tissue. (I and J) The Hippo component Tead4 is diurnally rhythmic in the intestine and its tumors (2-way ANOVA, f1, 168 = 5.125; P < .0001). (K and L) The Hippo target Ereg, is rhythmic in tumors but with a different rhythm than surrounding tissue (2-way ANOVA f5, 168 = 2.746; P = .02). (A and B) Pearson correlation showing the relationship between clock genes and Wnt pathway targets in nontransformed intestine (tissue) vs transformed tumors (tissue). Tissue normally shows a negative correlation (dark blue) between Bmal1 and Nr1d1, or Per2, and weaker correlation between Per2 and Nr1d1 and Wnt target genes (Axin2, cMyc, and Ccnd1). Tumors show loss of negative clock correlations, indicating weakened circadian transcription (where positive and negative regulators are expected to be antiphasic and thus negatively correlated). Increased positive correlation (dark red) between clock targets Per2 and Nr1d1 and Wnt target genes was observed in tumors. This indicates clock function was weakened, but daily rhythms in Wnt signaling were present in tumors. Error bars represent SEM.
A recent study showed that the clock component Nr1d1 is a tumor suppressor, whose activation in animal models dramatically reduced cancer. Further studies are needed to determine whether other clock genes have distinct effects on Apcmin tumor initiation. Our results add to a growing body of literature showing that clock gene disruption leads to cancer in a tissue-specific manner.

Recent studies have shown that tumors in the blood and brain both show and require circadian clock gene function. How is this possible? Is each tumorigenic event different, so that some tumors require clock function while others do not? Using circadian wild-type Apcmin mice, we found that in nearly all intestinal tumors, clock gene rhythms were present but at lower amplitude than the surrounding tissue in vivo (Figure 8A–F). Organoids derived from these tumors do not show self-autonomous clock gene transcription rhythms (Figure 4, Supplementary Table 1), showing that these cells lack a circadian oscillator. In vivo, circadian rhythmicity throughout the body is driven by hormones, responses to feeding, or other daily environmental changes experienced by the individual.52,78 The timing of food availability has been shown to improve circadian clock expression rhythms in tumor cells,79,80 and can even rescue Bmal1 loss of function in the liver.81 This explains how Apcmin tumor cells mimic the host circadian program in vivo, aligning their expression of circadian clock genes with the normal

Figure 10. Circadian rhythm loss potentiates tumor initiation. Control mice and Apcmin mice were tested on a normal 12-hour light/12-hour dark photoperiod (light dark cycle [LD]) vs constant light (LL, 700 Lux) (n = 5–6 per genotype). (A) Representative images of tumors found in LD and LL conditions. Scale bar: 200 μm. (B and C) Apcmin mice in LL develop significantly more large tumors greater than 1 mm in size (ANOVA, f(3, 20) = 20.58; P < .0001), and small tumors of less than 1 mm (ANOVA, f(3, 20) = 28.71; P < .0001) than LD mice, indicating that constant light recapitulates the Bmal1 mutant phenotype. No tumors were observed in control mice, under both LD and LL conditions, without the Apcmin allele present. Error bars represent SEM. (D and E) Representative images of total Yap-stained tumors in Apcmin (LD) and Apcmin (LL) show increased Yap+ nuclear signal in the cells of Apcmin−Bmal1−/−, which are present in higher numbers (t test, P = .0023). Scale bar: 50 μm. (F and G) Cell proliferation (Ki67+) is more intense and increased in Apcmin−Bmal1−/− crypts (t test P = .0341). Scale bar: 50 μm. (H and I) Proliferation also is increased in LL tumors (t test P = .0447). Histologic quantification shown for n = 10 crypts and n ≥ 3 animals per condition. Scale bar: 50 μm. All List of Antibodies Used error bars represent SEM.
physiological rhythms that surround them. This disrupted clock function in tumors is consistent with reports that cMyc, increased in Apc\textsuperscript{min} tumors (Figure 9G and H), inhibits circadian clock transcription.\textsuperscript{20,82,83} Indeed, we noted the presence of a cMyc transcriptional signature in Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} organoids compared with Apc\textsuperscript{min} control (Supplementary Table 5).

Despite their core clock deficiency, Apc\textsuperscript{min} tumor organoids showed 24-hour variation in hundreds of transcripts in vitro, and Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} tumor organoids showed 24-hour variation in another set of transcripts (Supplementary Table 1). We cannot rule out that some of these 24-hour variations in organoids without a functional clock are false positives caused by genome-wide testing of thousands of genes; nevertheless, the ability of Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} organoids to show rhythms is a curious phenomenon, previously found to occur in both transcription and metabolism.\textsuperscript{84–86} Further experiments are required to classify the nature of these self-sustained oscillations in the absence of the core transcriptional oscillator, and their possible biological implications in organoid culture. In contrast to the tumor epithelium, the nontransformed epithelium shows robust circadian clock activity\textsuperscript{87,88} (Figures 8 and 9). Using organoid cultures, we detected 41 self-sustaining rhythmic genes dependent on Bmal1\textsuperscript{46} (Figure 3). Other studies have detected many more rhythmic genes in the intestine.\textsuperscript{89–93} Our RNA sequencing approach differed from these studies in several important respects: (1) testing only the cells of the intestinal epithelium, enriched for crypt precursors present in the first 2 days of organoid cultures; (2) using the Bmal1\textsuperscript{−/−} mutant to distinguish putative core clock targets from rhythmic but non–Bmal1-regulated genes; (3) testing circadian gene expression under free-running in vitro conditions to exclude environmental responses (such as photoperiod or feeding) from self-sustaining circadian rhythmicity; and (4) performing high-resolution transcriptomic analysis, which would exclude stochastic non-circadian changes in daily gene expression. We have attempted to follow the guidelines proposed for optimal detection of circadian transcription,\textsuperscript{56} and it is apparent that epithelial crypt cells have relatively few rhythmic circadian gene targets. Among these are genes known to be involved in ISC biology, suggesting the circadian clock does indeed regulate aspects of tissue renewal of interest for future study. An important caveat to our analysis is that the gene expression of differentiating cells may obscure circadian expression and therefore our analysis should not be considered exhaustive. Additional tests of specific differentiated cell types in organoids, or longer (ie, 48-hour) timelines, would be informative.

Although our study has focused on the epithelium, other aspects of physiology that are dysregulated in Bmal1\textsuperscript{−/−} mice\textsuperscript{51} also may contribute to tumor initiation. The circadian clock is known to regulate many aspects of inflammation,\textsuperscript{94} and we report that dysfunction in immune system activity may be an additional factor (Figure 6D–F), when the clock is disrupted throughout the body. It has been shown that glucocorticoid rhythms in the body suppress tumor growth.\textsuperscript{95} Our analysis, showing lower glucocorticoid levels in Bmal1\textsuperscript{−/−} null genotypes (Figure 1A), also supports the additional contribution of disrupted endocrine homeostasis to tumorigenesis in Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} mice. Both inflammatory and metabolic contributions to tumor growth during circadian disruption will be important to test to distinguish the effects of these contributing factors.\textsuperscript{36}

This study reports circadian clock regulation of the Hippo pathway. Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−} organoids show higher Yap/Tead target genes (Figure 5B and D); Apc\textsuperscript{min} + Bmal1\textsuperscript{−/−}–mutant, Apc\textsuperscript{min} Bmal1 intestinal cKO, and Apc\textsuperscript{min} circular-disrupted (LL) tumors show more Yap activity, and higher Tead4 and Ereg expression (Figures 7 and 10). These changes are associated with concomitant lower levels of Wnt target genes in both organoids and tumors. Wnt pathway activation is the primary cause of Apc\textsuperscript{min}-induced tumorigenesis,\textsuperscript{36} but the Hippo pathway is implicated in Apc\textsuperscript{min} tumorigenesis as well.\textsuperscript{43} Studies have shown that these pathways may be mutually antagonistic, and consistent with this notion we note that Wnt and Hippo target-expressing cells generally are separate (Figure 7F–I). We therefore hypothesize that Bmal1 negatively regulates the Hippo signaling pathway in the tumor epithelium, and Wnt pathway regulation emerges as a secondary effect. Intestinal regeneration has been proposed to involve the reprogramming of a fetal-like state with high Yap activity.\textsuperscript{42,43,97} Crypt cell populations readily dedifferentiate during regeneration,\textsuperscript{48} where Yap activity is precisely controlled before the system returns to a primarily Wnt-driven, self-renewal state.\textsuperscript{65} An endogenous timekeeper such as the circadian clock would be an ideal system to control the timing of regenerative pathway responses, thereby dictating the time when Wnt and Hippo signaling pathways are engaged during stress and regeneration. This is analogous to the role of the circadian clock in regulating signaling pathways in epidermal stem cells.\textsuperscript{98} Understanding the circadian regulation of ISC signaling will help develop cancer-preventing measures, as well as providing insight into the diagnosis and treatment of colorectal cancers in individuals subject to shift work or other forms of circadian disruption.

Methods

Animal Housing and Breeding

Mice were housed on a 12-hour light/12-hour dark cycle with ad libitum food in a barrier facility. Apc\textsuperscript{min} mice (#002020; Jackson Labs, Bar Harbor, ME) were crossed with Bmal1\textsuperscript{−/−} null mutant mice (#009100; Jackson Labs). Progeny were back-crossed for 7 generations to generate a genetically pure strain before experiments began. Bmal1\textsuperscript{−/−} mice (#007668; Jackson Labs) were crossed with Villin\textsuperscript{cre/+} mice (#021504; Jackson Labs), or Apc\textsuperscript{min} mice, then the 2 strains were crossed to obtain the Apc\textsuperscript{min/+}; Bmal1\textsuperscript{−/−} , Bmal1\textsuperscript{−/−} × VIl\textsuperscript{cre/+}, or Bmal1\textsuperscript{−/−} × Bmal1\textsuperscript{−/−} for the cKO experiments. Genotyping was performed according to protocols available at Jackson Labs (www.jax.org). All animals were tested for tumor initiation at 1 or 3 months of age. Mice maintained under constant light (LL) were housed in these conditions from 21 days of age to 3 months of age. Light levels were approximately 700 Lux, measured at the
performed by Eve Technologies (Calgary, Alberta, Canada).

Tnf and interleukin-6 blood cytokine measurements were performed on mice euthanized by cervical dislocation at the times indicated in the figure panels. Intestines were then removed and flushed with ice-cold, phosphate-buffered saline to remove feces. Histologic analysis and tumor counting, the entire length of the small intestine was fixed in 4% paraformaldehyde (PFA) + phosphate-buffered saline (PBS) (Electron Microscopy Sciences, Hatfield, PA) for 2 hours at room temperature. The intestine was subdivided into 4 regions of equal length longitudinally: the proximal intestine closest to the stomach containing duodenum and jejunum, the distal-medial region containing the jejunum, the proximal region containing the jejunum, and ileum, and the distal region containing the ileum. In Figure 1 and Supplementary Figure 1, the 2 medial regions are combined. Tumors were counted visually and tumors greater than 1 mm were scored by comparing size with a 1-mm diameter glass capillary tube. Tissue was sectioned at 10 μm and frozen at -80°C. Tissue was sectioned at 10 μm using a Leica CM 1950 Cryostat (Leica Biosystems, Concord, Ontario). H&E staining and Alcian Blue staining was performed as per previous protocol.46 Immunohistochemistry was performed as previously described.46 Tumor dysplasia was blindly evaluated using a previously published scoring system: the number of adenomas and crypt architecture was assessed in the distal, medial, and proximal sites in the tissue, and the alteration in mucosa architecture, epithelial changes, hyperplasia, crypt abscesses, and crypt loss were scored from 0 to 4 (most severe) score.98,99 Corticosterone levels were determined by enzyme-linked immunosorbent assay (ADI-900-097; Enzo Life Sciences, Farmingdale, NY) in duplicate according to kit instructions at a 1:80 dilution with 1% steroid displacement buffer. Samples were assayed in 2 groups, with intra-assay variation of 7.3% and 7.7%, and interassay variation of a pooled mouse control was 9.5% and 9.3%.

**Tissue Collection**

All animals were killed using CO2, followed by cervical dislocation at the times indicated in the figure panels. Intestines were then removed and flushed with ice-cold, phosphate-buffered saline to remove feces. Histologic analysis and tumor counting, the entire length of the small intestine was fixed in 4% paraformaldehyde (PFA) + phosphate-buffered saline (PBS) (Electron Microscopy Sciences, Hatfield, PA) for 2 hours at room temperature. The intestine was subdivided into 4 regions of equal length longitudinally: the proximal intestine closest to the stomach containing duodenum and jejunum, the distal-medial region containing the jejunum, the proximal region containing the jejunum, and ileum, and the distal region containing the ileum. In Figure 1 and Supplementary Figure 1, the 2 medial regions are combined. Tumors were counted visually and tumors greater than or less than 1 mm were scored by comparing size with a 1-mm diameter glass capillary tube. Tissue was frozen in Tissue-Tek OCT (Optimal Cutting Temperature) Compound (#62550-12; Electron Microscopy Sciences). Tissue used for RNA-Scope (ACD, Newark, CA) analysis was fixed in 4% PFA + PBS for 24 hours at 4°C. Tissue used for gene expression analysis was placed in RNAlater buffer (Qiagen, Germantown, MD) at 4°C overnight (O/N), then stored at -80°C. Blood samples were collected in heparinized tubes, centrifuged to remove blood cells at room temperature, and then stored at -80°C until analysis. Tnf and interleukin-6 blood cytokine measurements were performed by Eve Technologies (Calgary, Alberta, Canada).

**Staining and Western Blot**

Tissue was washed 3 × in PBS and placed in 30% sucrose in PBS O/N. Tissue then was equilibrated in OCT for 1 hour and frozen at -80°C. Tissue was sectioned at 10 μm using a Leica CM 1950 Cryostat (Leica Biosystems, Concord, Ontario). H&E staining and Alcian Blue staining was performed as per previous protocol.46 Immunohistochemistry was performed as previously described.46 Tumor dysplasia was blindly evaluated using a previously published scoring system: the number of adenomas and crypt architecture was assessed in the distal, medial, and proximal sites in the tissue, and the alteration in mucosa architecture, epithelial changes, hyperplasia, crypt abscesses, and crypt loss were scored from 0 to 4 (most severe) score.98,99 3,3'-Diaminobenzidine tetra hydrochloride staining was performed using horseradish peroxidase/3,3'-diaminobenzidine tetra hydrochloride (ABC) Detection IHC Kit as per the manufacturer’s protocol (ab64261; Abcam). RNA in situ probe hybridization and detection was performed using the RNAscope method according to the manufacturer’s protocol (ACD, Newark, CA). Antibodies used are shown in Table 1.

Tumors were microdissected, cut into small pieces, and then lysed in RIPA buffer containing Pierce Protease and Phosphatase Inhibitor (Pierce, Waltham, MA). Electrophoresis was performed on Mini-PROTEAN TGX Stain-free gels (Bio-Rad, Hercules, CA) and Western blot on polyvinylidene difluoride (Thermo Scientific, Waltham, MA). Western blot

---

**Table 1. List of Antibodies Used**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Assay</th>
<th>Supplier</th>
<th>Product number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylated-histone H3 (Ser10)</td>
<td>1:1000</td>
<td>IF</td>
<td>Upstate/Millipore (Oakville, Ontario)</td>
<td>06-570</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>1:200</td>
<td>IF</td>
<td>Cell Signaling Tech (Whitby, Ontario)</td>
<td>9661S</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1:200</td>
<td>IF</td>
<td>Agilent/Dako (Santa Clara, CA)</td>
<td>A0909</td>
</tr>
<tr>
<td>Nrd1</td>
<td>1:250</td>
<td>IF</td>
<td>Abnova (Taipei City, Taiwan)</td>
<td>H00009572 M02</td>
</tr>
<tr>
<td>Yap (D841X) XP (R)</td>
<td>1:100</td>
<td>DAB</td>
<td>Cell Signaling Tech</td>
<td>140745</td>
</tr>
<tr>
<td>1:1000</td>
<td>WB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td>1:250</td>
<td>DAB</td>
<td>Abcam (Toronto, Ontario)</td>
<td>Ab32572</td>
</tr>
<tr>
<td>Ki67</td>
<td>1:100</td>
<td>DAB</td>
<td>Abcam</td>
<td>Ab16667</td>
</tr>
<tr>
<td>CD45</td>
<td>1:200</td>
<td>IF</td>
<td>Abcam</td>
<td>ab10558</td>
</tr>
<tr>
<td>Nonphosphorylated YAP (Ser127) (E6U82) rabbit mAb</td>
<td>1:1000</td>
<td>WB</td>
<td>Cell Signaling Tech</td>
<td>29495S</td>
</tr>
<tr>
<td>Tubulin</td>
<td>1:10,000</td>
<td>WB</td>
<td>Sigma</td>
<td>T5168</td>
</tr>
<tr>
<td>DK α-rabbit-HRP</td>
<td>1:10,000</td>
<td>WB</td>
<td>Jackson Immunoresearch (West Baltimore Pike, PA)</td>
<td>711-035-152</td>
</tr>
<tr>
<td>DK α-mouse-HRP</td>
<td>1:10,000</td>
<td>WB</td>
<td>Jackson Immunoresearch</td>
<td>715-035-150</td>
</tr>
</tbody>
</table>

DAB, 3,3′-diaminobenzidine tetra hydrochloride; DK, Donkey; HRP, horseradish peroxidase; IF, Immuno-Fluorescence; mAb, Monoclonal Antibody; WB, Western Blot; Yap, Yes-associated protein 1.
were blocked for 30 minutes with 5% milk in PBS and 0.1% Tween 20. Primary antibodies were incubated overnight at 4°C. Secondary antibodies were incubated for 1 hour at room temperature. Horseradish peroxidase was developed using Super Signal Femto (Thermo Scientific) and imaged on a Bio-Rad gel Imager. All samples were collected at Zeitgeber Time 8. The antibodies used are shown in Table 1.

**Microscopy**

Samples were imaged using a slide scanner (Axio Scan.Z1; Zeiss, Toronto, Canada). The tumor area was quantified using Zen Blue Software (Zeiss). Organoid cloning images were obtained with a 5× objective on an AXIO Vert.A1 microscope (Zeiss). Images were processed using Adobe (San Jose, CA) Photoshop CS5. Antibody-positive cells were quantified as the total number of labeled cells or positive nuclei. Ten crypts were sampled per animal for each intestinal region (proximal, medial, and distal) for quantification of PHH3, lysozyme, cleaved caspase-3, Ki67, Nr1d1, Yap, and Axin2. Regions were averaged together for results shown if no significant differences were observed.

**RNA Purification**

Tissue samples were homogenized using a bullet blender (Next Advance, Troy, NY), and RNA was isolated using the RNeasy mini Purification Kit (Qiagen). Complementary DNA was produced using the iScript RT Supermix (Bio-Rad) according to the manufacturer’s protocol.

**Quantitative Polymerase Chain Reaction**

Transcript levels were quantified using iTaq Universal SYBR Green Supermix (Bio-Rad) on a ViiA7 real-time polymerase chain reaction machine (Thermo Fisher Scientific). Primer sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and validated at r2 > 0.98, with an efficiency of approximately 80–110. The primers used are listed in Table 2.

**Organoid Experiments**

Intestinal organoids were derived from mice as previously reported. For the 24-hour RNA sequencing, organoid biological replicates were collected every 2 hours (n = 2 derived lines of each of the 4 genotypes). For the 48-hour analyses, organoid biological replicates (n = 3 derived lines of each of the 4 genotypes) were collected every 4 hours. RNA sequencing experiments were performed by splitting organoids, allowing a 12-hour recovery, and then 1-hour treatment with 0.1 μM dexamethasone (Sigma, Oakville, Ontario) in base media containing epidermal growth factor. Media then was replaced with fresh organoid culture media. Samples were collected from 24 hours after dexamethasone treatment, every 2 hours, and immediately lysed in RLT (Qiagen) + 1% β-mecaptoethanol (M6250; Sigma Aldrich) and flash frozen in liquid nitrogen. For organoid cloning assays, 5 large organoids were selected from 1 animal of each genotype and dissociated for 10 minutes using 100 μL TrypLE Express (12605010; Thermo Fisher) + 1:1000 Y-27632 (72304; Stem Cell Tech, Vancouver, British Columbia). Enzymatic dissociation was quenched with 100-μL base organoid media containing epidermal growth factor + 1:1000 Y-27632. Single cells then were plated in ENR (EGF, Noggin) / EN (EGF, Noggin) media + Y-27632 for 1 day. The following day, Y-27632 was removed and replaced with fresh ENR/EN media. Fast-growing Apcmin tumor organoids were quantified 5 days later, whereas slower-growing nontransformed organoids were quantified 15 days later in all experiments. In all experiments, organoid media was changed every 2–5 days, depending on need. Yap chemical inhibition was performed using 3 mmol/
Table 3. Details of Statistical Tests

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Two-way ANOVA</td>
<td>$f_{(8, 52)} = 2.766; P = .0208$</td>
</tr>
<tr>
<td>C</td>
<td>One-way ANOVA</td>
<td>$f_{(2, 48)} = 80.56; P &lt; .0001$</td>
</tr>
<tr>
<td>D</td>
<td>One-way ANOVA</td>
<td>$f_{(2, 47)} = 57.41; P &lt; .0001$</td>
</tr>
<tr>
<td>F</td>
<td>Two-way ANOVA</td>
<td>$f_{(2, 399)} = 5.936; P = .0029$</td>
</tr>
<tr>
<td>G</td>
<td>Two-way ANOVA</td>
<td>$ZT12 P = .0030$</td>
</tr>
<tr>
<td>I</td>
<td>Unpaired t test</td>
<td>$P = .1036$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 2</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Unpaired t test</td>
<td>$P = .0012$</td>
</tr>
<tr>
<td>D</td>
<td>Unpaired t test</td>
<td>$P = .0166$</td>
</tr>
<tr>
<td>F</td>
<td>Two-way ANOVA</td>
<td>$ZT12 P = .00246$</td>
</tr>
<tr>
<td>G</td>
<td>Unpaired t test</td>
<td>$P = .031$</td>
</tr>
<tr>
<td>I</td>
<td>Unpaired t test</td>
<td>$P = .1409$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>MetaCycle2d</td>
<td>Nrd1d ($P = 4.9 \times 10^{-8}; q = 0.00039$), Cry1 ($P = 3.8 \times 10^{-8}; q = 0.00039$), Bmp3 ($P = .00012; q = 0.057$), Ephb3 ($P = 8.0 \times 10^{-6}; q = 0.0084$), Kit ($P = .00016; q = 0.067$), Tead4 ($P = 1.09 \times 10^{-6}; q = 0.0098$), Nrd1d ($f_{(12, 37)} = 5.393; P &lt; .0001$), Cry1 ($f_{(3, 37)} = 413.1; P &lt; .0001$), Tead4 ($f_{(12, 37)} = 15.36; P &lt; .0001$).</td>
</tr>
<tr>
<td>D</td>
<td>Two-way ANOVA</td>
<td>GoLab ($P = 12.0 \times 10^{-6}; q = 0.015$), Dolk ($P = 2.83 \times 10^{-6}; q = 0.015$), RNF128 ($P = 5.81 \times 10^{-6}; q = 0.015$), Mlxipl ($P = 4.26 \times 10^{-7}; q = 0.0053$), Fam76b ($P = 1.44 \times 10^{-6}; q = 0.0055$), Nrd1d ($f_{(12, 37)} = 5.393; P &lt; .0001$), Cry1 ($f_{(3, 37)} = 413.1; P &lt; .0001$), Tead4 ($f_{(12, 37)} = 15.36; P &lt; .0001$).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Gene-wise 1-way ANOVA (4 genotypes) with BH correction</td>
<td>$f_{(3, 84)} &gt; 2.75; P &lt; .047; q &lt; 0.05; P &lt; .00357; q &lt; 0.05$</td>
</tr>
<tr>
<td>B</td>
<td>Gene-wise 1-way ANOVA (4 genotypes) with BH correction</td>
<td>$f_{(3, 84)} &gt; 2.75; P &lt; .047; q &lt; 0.05; P &lt; .002815; q &lt; 0.05$</td>
</tr>
<tr>
<td>C</td>
<td>GO analysis: DAVID 6.8</td>
<td>$P &lt; .0002; Benjamini &lt; 0.05$</td>
</tr>
<tr>
<td>D</td>
<td>ES</td>
<td>ES, 0.44; NES, 2.083; $P = .000; q = 0.000; P = .000$</td>
</tr>
<tr>
<td>G</td>
<td>Unpaired t test</td>
<td>$P = .0004$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 5</th>
<th>Test</th>
<th>Panel</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>One-way ANOVA</td>
<td>$f_{(3, 10)} = 19.55; P = .0002; P = .0068$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Tukey post-test</td>
<td>$f_{(2, 49)} = 4.69; P = .0144; P = .0272; P = .0044$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Unpaired t test</td>
<td>$P = .0004$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 6</th>
<th>Test</th>
<th>Panel</th>
<th>Test</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>One-way ANOVA</td>
<td>$f_{(3, 41)} = 11.48; P = .0026; P = .0029$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Tukey post-test</td>
<td>$f_{(3, 65)} = 6.824; P = .005; P = .0182$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Unpaired t test</td>
<td>$P = .027$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
L. Verteporfin (AOB5623; Aobious, Gloucester, MA), Tead inhibition using 3 mmol/L TED-347 (AOB17707; Aobious), and 1:750 dimethyl sulfoxide vehicle control (D2650; Sigma) was applied to organoid media.

**RNA Sequencing and Gene Expression Analysis**

A total of 1 μg RNA per sample was prepared at the Donnelly Sequencing Centre (http://ccbr.utoronto.ca/donnelly-sequencing-centre) for RNA integrity assessment and sequencing. Libraries were prepared using 96 NEB–NEBNext Ultra II Directional RNA Library Prep Kit including quality control and DNase treatment (#E7765; New England Biolabs, Ipswich, MA). Sequencing was performed on a 1 Flowcell, Illumina NovaSeq6000 (Illumina), S2; 100 cycles; v1 chemistry read 1, 101 bp; index read 1, 8 bp; and index read 2, 8 bp. Raw transcriptome sequencing reads were aligned against GRCm38 and Ensembl release 96 transcript annotations using STAR v2.5.100 RSEM v1.3.0.101 was used for transcript quantification. Libraries were excluded from further analyses in the case of low-quality alignment (Supplementary Table 4). Transcripts per million values for all remaining libraries in each genotype were merged into 1 expression table file. Quantile normalization was performed for each expression table using the preprocessCore package. Rhythmic analysis and differential expression analysis were performed as follows. First, genes with null expression in at least 1 sample within a table were excluded from that table. Rhythmicity for the remaining transcripts then was evaluated with MetaCycle,27 and we considered all transcripts with a base expression greater than 0.5, relative amplitude greater than 0.05, and a BH q value less than 0.1 as rhythmic. The number of rhythmic transcripts was as follows: 41 in control organoids, 0 in Bmal1 mutant, 238 in Apcmin control, and 263 in Apcmin + Bmal1 mutant (Supplementary Table 1). We next evaluated gene-by-gene (n = 17,543) the differences in overall expression across 24 hours (all time points) between genotypes/expression tables, using analysis of variance (ANOVA), followed by the post hoc Tukey test. For each gene, pairwise expression fold changes between genotypes were calculated as the ratio between the average 24-hour expression in the first genotype and the average in the second genotype. With a Tukey q value less than 0.05 and |log2(fold change)| greater than 1.5, the number of differentially expressed genes was as follows: 111 between control and Bmal1 mutant, 6405 between control and Apcmin control, and 2398 between Apcmin control and Apcmin + Bmal1 mutant (Supplementary Table 1).
control and Apemin + Bmal1 mutant (Supplementary Table 2). GSEA was conducted in the GSEA 4.1.0 software downloaded from https://www.gsea-msigdb.org/gsea/index.jsp. Genes selected to be input into GSEA had to be expressed in two thirds of the samples (Supplementary Table 5). The gene set (size = 425) for GSEA analysis was generated using genes found to be up-regulated 2-fold or greater in RNA sequencing data set.

Statistics

Statistical analysis was performed using Prism 8 (GraphPad, San Diego, CA). An unpaired t test, 1-way ANOVA, or 2-way ANOVA were used with a post-test (Table 3). RNA sequencing data analyses were performed in R. MetaCycle was applied using JTK and LS algorithms and the Fisher method for P value integration. For comparisons between genotypes, the P values from ANOVA and the post hoc Tukey tests were corrected with the BH method. In these tests, genes that were absent in any 1 genotype were assigned the lowest expression value found in the expression tables. Pearson heatmaps were plotted using the Python 3.6-Seaborn library based on the Pearson correlation between the participant genes. Cubic spline interpolation was used to model the growth of a gene throughout time points (Figures 8 and 9). The splines are natural where the first derivative \( \partial_1 \) and the second derivative \( \partial_2 \) both equal 0 at each time point. Implementation of the cubic spline interpolation was performed using Matlab 2013a.

References


51. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the


57. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem 2002;277:33275–33283.


2021 Circadian Disruption Increases Tumorigenesis 25


