J. Biochem. 2009;145(3)289–297
doi:10.1093/jb/mvn167

β-Catenin Induces β-TrCP-Mediated PER2 Degradation Altering Circadian Clock Gene Expression in Intestinal Mucosa of *Apc*$^{Min/+}$ Mice

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Received September 10, 2008; accepted November 21, 2008; published online December 23, 2008

Proliferation of intestinal epithelial cells is rhythmic throughout the day. This temporal organization occurs through the interaction between the endogenous peripheral circadian clock and pathways controlling cell cycle progression. *Per2*, a core clock gene with tumour suppresser function, is critical to clock function and to the regulation of cellular proliferation. Circadian disruption, which increases colon cancer incidence, may do so by deregulating clock controlled epithelial cell proliferation. Increased expression of β-catenin is a contributing cause of most familial and spontaneous human colon cancer and the cause of multiple intestinal neoplasia of the *Apc*$^{Min/+}$ mouse. Here we report that increased β-catenin destabilizes PER2 clock protein by inducing β−TrCP, an F-box protein of SCF ubiquitin E3 ligase. In the intestinal mucosa of the *Apc*$^{Min/+}$ mouse, the decrease in PER2 protein levels is associated with altered circadian rhythms of clock genes, *Per1* and *Per2*, and clock controlled genes, *Dbp* and *Wee1*. These findings suggest that disruption of the peripheral intestinal circadian clock may be intimately involved in β-catenin induced intestinal epithelial neoplastic transformation in both mouse and man.

Key words: circadian, colon cancer, *Per2*, β-catenin, β-TrCP.

Abbreviations: *APC*, adenomatous polyposis coli gene; *Apc*$^{Min/+}$, heterozygous Min (multiple intestinal neoplasia) mutation of the *APC* gene; ATCC, The American Type Culture Collection; BMAL1/*Bmal1*, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like core circadian clock protein/gene; CHX, cycloheximide; CLOCK, circadian locomotor output cycles kaput; CRY/*Cry*, Cryptochrome protein/gene; DD, 24h constant darkness lighting condition; *Dbp*, albumin D-box binding protein gene; FBS, fetal bovine serum; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3-beta; HALO, Hours After Lights On; LD, 12h light 12h dark lighting condition; NP-40, nonyl phenoxylpolyethoxylethanol; NPAS2, neuronal PAS domain protein 2; PBS, phosphate buffered saline; PER/*Per*, Period protein/gene; SCF, Skp1-Cul1-F-box-protein; SCN, suprachiasmatic nuclei; TS, thymidylate synthase; VEGF, vascular endothelial growth factor; β-TrCP, beta-transducin repeat containing protein.

INTRODUCTION

In mammals, most physiological and pathological processes oscillate with an about 24 h period, in tune with their circadian environments. Circadian clocks beat within humans bearing cancer and within in their cancers (*1–6*). This temporal organization is accomplished through the interaction of endogenous central and peripheral circadian clocks which modulate tissue specific processes. The clock is composed of a set of clock genes forming two interlocked positive and negative feed back loops. Two transcriptional factors, CLOCK (or NPAS2) and BMAL1 activate the transcriptions of *Period* (*Per1*, *2*) and *Cryptochromes* (*Cry1*, *2*) genes. Then PER and CRY proteins form dimers and suppress their own transcription. In this process, the rhythmic expression of *Period* genes is essential for generating a functional circadian clock (*7–10*). The circadian clockwork is also controlled by posttranslational modification and stability of clock proteins. The SCF ubiquitin ligases are involved in the circadian clockwork, as they target PER and CRY proteins for timely degradation (*10–13*).

The circadian clock also influences the rhythmic expression of key tissue processes, such as cell proliferation (*14*). Examples of such clock-proliferation connections include circadian rhythms such as vascular endothelial growth factor (VEGF), which influences tumour growth; Wee-1 which gates cell mitosis; p16 and p21 each of which arrest cell cycle progression; and Cyclin D, Cyclin E and thymidylate synthase that, respectively, regulate cell cycle progression and provide essential thymidine (*1*, *15–17*).

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The core circadian clock gene *Per2* also has unique tumour suppressor functions (*18*, *19*). *Per2* gene is mutated in human breast cancers and the expression of PER2 protein is decreased in colon cancers (*20–22*). The tumour suppressor function of *Per2* works, in part, through the clock's control of the circadian organization of cancer growth. Down regulation of *Per2* within cancer cells results in increased tumour growth rate, at some but not other times of day (*23*). The circadian gating of cancer cell proliferation, thereby, has implications for the optimal timing of therapeutic agents targeting cancer cell proliferation (*24*). Loss of *Per2* increases *Apc* mutation-mediated intestinal neoplastic transformation (*25*).

Most human colorectal cancers demonstrate enhanced expression of β-catenin, which constitutively activates the Wnt signaling pathway and increases expression of downstream genes, such as *c-Myc, Cyclin D* and *VEGF*, which, in turn, promote cell proliferation. In the absence of Wnt signaling, β-catenin associates with adenomatous polyposis coli (APC) protein and is subsequently phosphorylated by glycogen synthase kinase 3β (GSK-3β). The phosphorylated β-catenin protein is then ubiquitinated by ubiquitin E3 ligase $SCF^{\beta\text{-TrCP}}$ and degraded by 26S proteasome (*26–28*). β-TrCP, an F-box protein, which determines the substrate specificity of SCF ligase, targets a variety of proteins modulating proliferation, apoptosis and DNA damage response pathways (*29*). β-TrCP also regulates the molecular circadian clockwork by targeting PER1 and PER2 for their ubiquitin mediated degradation (*11*, *12*). Recent studies showing upregulated β-TrCP in colon cancer (*30*) prompted us to ask whether the expression of clock genes and clock-controlled cell cycle genes are altered in the small intestinal mucosa of cancer prone $Apc^{Min/+}$ (multiple intestinal neoplasia) mice.

Here we report, β-catenin enhances PER2 protein degradation in colon cancer cells by up regulating β-TrCP. In the intestinal mucosa of $Apc^{Min/+}$ mice, the overall level of PER2 protein is much lower and its daily rhythm is lost compared with wild type. In addition, the RNA expression of *Period* genes and other key proliferation related clock-controlled genes are altered.

METHODS AND MATERIALS

Animal Housing Condition and Tissue Processing—Wild-type C57BL/6 and $Apc^{Min/+}$ were kept under 12 h light 12 h dark (LD) conditions with food and water freely available. When they reached 15 weeks of age, tissue samples were obtained from eight mice at one of six equispaced times around the clock referenced as HALO (Hour After Light On, Light on at HALO 0 and off at HALO 12). Small intestinal mucosa was collected as previously described (*31*). Intestinal polyps in $Apc^{Min/+}$ mice were removed using a dissecting microscope. Experiments were performed in compliance with NIH and VA guide lines for care and use of laboratory animals.

Measurement of Circadian Locomotor Activity—Mice at 13 weeks of age were singly housed in cages with a running wheel with a free access to diet and water under LD conditions. After adaptation to running wheels, the lighting schedule was changed from LD to 24 h dark/dark (DD) condition to assess the free running circadian period (a measure of central clock function). Circadian locomotor activity (number of revolutions/5 min) was recorded through VitalView software (Minimitter Co., OR) via a magnetic sensor attached to the wheel.

Cell Culture and siRNA—Human colon cancer cell lines HCT116 and SW480 and human 293T cells were obtained from ATCC. They were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) under the condition of 5% CO_2 at 37°C. For siRNA transfection, cells were plated in six-well plates the day before to reach 30% of confluency by the time of transfection. siRNA (100 pmol) was transfected by Lipofectmine2000 (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacture. The knock down effect was examined after 72 h of incubation. The sequences of RNA oligos were as follows:

GFP(control): 5′-rArArGrGrCrArArGrCrUrGrArCrCrCrUrGrArATT

5′-rUrUrCrArGrGrGrUrCrArGrCrUrUrGrCrCrUrUTT

β-catenin: 5′-rGrCrUrGrGrUrGrGrArArUrGrCrArArGrCrUrUTT

5′-rArArGrCrUrUrGrCrArUrUrCrCrArCrCrArGrCTT.

β-TrCP: 5′-rGrCrGrArArUrUrCrUrCrArCrArGrGrCrCrArUTT

5′-rArUrGrGrCrCrUrGrUrGrArGrArArUrUrCrGrCTT

Protein Half-Life Assay—HCT116 cells were treated with control or β-catenin siRNA. Three days later, 50 μg/ml of cycloheximide (CHX) was added to block new protein synthesis. Samples were taken at the indicted time and total proteins were extracted for western blotting. For co-expression experiment, 293T cells were transfected with V5-*Per2* and an empty vector, with FLAG-β-catenin, FLAG-β-catenin S37A ($SCF^{\beta\text{-TrCP}}$ resistant mutant), or with FLAG-β-catenin and β-TrCP siRNA. The half lives of V5-PER2 proteins were compared after 48 h.

Western Blotting—Cells were washed with PBS once and then total protein extracts were obtained by incubating in NP40 buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris–Cl pH 7.4, protease inhibitors). The soluble proteins from small intestinal mucosa were isolated as described previously (*31*). Proteins were then separated by standard SDS–PAGE using and transferred to Nitrocellulose membranes. The anti-PER2 antibody was from Alpha Diagnostic (San Antonio, TX). The anti-β-TrCP antibody was from Invitrogen. The other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

RT-PCR Quantification—Total RNA was isolated from tissues using Trizol and reversely transcribed by M-MLV reverse transcriptase (Invitrogen) according to the provided protocol. Real-time quantitative PCR analyses were performed on an iCycler iQ PCR system (BioRad Lab). PCR reagents were purchased from BioRad Lab. Relative amount of target gene expression was computed with respect to the endogenous reference (*Gapdh* gene), expressed as ratio of target gene/*Gapdh*. The primer pairs for the amplification of each gene were listed in Table 1.

Data Analysis—To characterize sinusoidal properties of circadian data, a cosine curve was fit by the method of least squares and tested for statistical significance by rejection of the zero amplitude hypothesis using Chronolab v4.6 (Bioengineering & Chronobiology Laboratory, Pontevedra, Spain). Cosinor analysis proves

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Table 1. **Primers used for real time PCR amplification of mouse gene**

Gene	Forward primer	Reverse primer
Gapdh	5′-CATGGCCTTCCGTGTTCCTA-3′	5′-CCTGCTTCACCACCTTCTTGA-3′
Per1	5′-CAGCTGGGCCGGTTTTG-3′	5′-CACTTTATGGCGACCCAACA-3′
Per2	5′-GAGCAGGTTGAGGGCATTAC-3′	5′-TGGAGGCCACTTGGTTAGAG-3′
Bmal1	5′-ACAACGAGGGCTGCAACCT-3′	5′-CCCGTTCGCTGGTTGTG-3′
Rev-erbα	5′-AACCTCCAGTTTGTGTCAAGGT-3′	5′-GATGACGATGATGCAGAAGAAG-3′
Dbp	5′-GCCCACCTGGTACAGAAGGA-3′	5′-TCAAGCAGCTGTCTCTTTGCA-3′
Wee1	5′-GAAACAAGACCTGCCAAAAGAA-3′	5′-GCATCCATCTAACCTCTTCACAC-3′

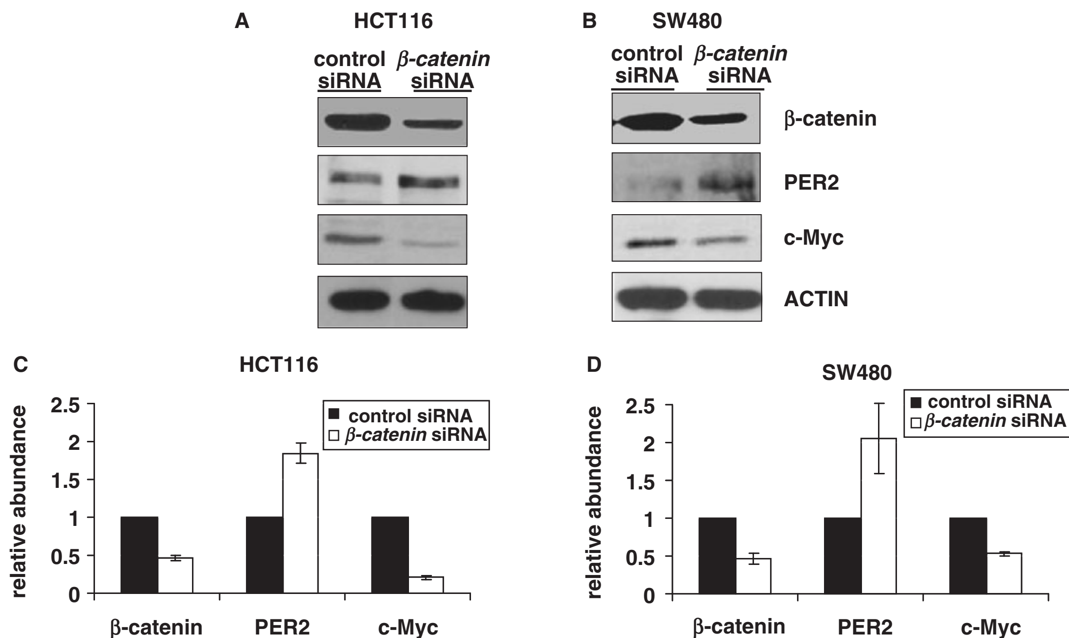

Fig. 1. **Down-regulation of β-catenin increases PER2 protein.** Immunoblot assay of total cell extracts of HCT116 (A) and SW480 (B) treated with control or β-catenin siRNA. The relative amounts of protein were quantified from three independent experiments (C and D) and the levels of the controls were set as 1. Values are means and standard errors of repeat experiments.

or disproves a significant daily rhythm and generates mesor (24 h adjusted mean), amplitude (1/2 peak-trough difference), percent rhythm (pr, % variation explained by daily rhythm) and acrophase (time of peak values). Cosine curves for 24 h period were fit to these data. Bingham tests were used to contrast two rhythms to discern in there were significant differences in amplitude, mesor or acrophase between the rhythms. Repeated measures analysis of variance (ANOVA) was performed to quantify time effect by comparing means at each six circadian HALOs with one another; significance was determined at 0.05 error probability (SPSS, SAS software). Two-tailed testing was always employed.

RESULTS

Down Regulation of β-Catenin in Human Colon Cancer Cells Increases PER2 Protein—Most colon cancers are, in part, caused by elevated level of β-catenin protein. Since β-catenin increases the expression of β-TrCP, which potentially could target PER2 for its ubiquitin-mediated degradation, we examined whether β-catenin affects PER2 expression in colon cancer. In colon cancer cells, HCT116 and SW480, endogenous β-catenin protein is elevated due to a mutation of β-catenin (HCT116) or *APC* (SW480) genes. In both cell lines, when β-catenin is down regulated by siRNA, PER2 protein levels are increased (Fig. 1). Knocking down β-catenin in these cells also decreases c-Myc protein (Fig. 1). This is consistent with the finding that *c-Myc* expression is activated by the β-catenin/TCF4 transcriptional factor (*32*).

β-Catenin Regulates PER2 Protein Stability—Since β-catenin and PER2 share degradative machinery (*12*), we hypothesized that β-catenin might regulate PER2 protein stability. We reasoned that this could be mediated by β-catenin-enhanced expression of β-TrCP, an F-box protein targeting both β-catenin and PER2 protein for polyubiquitination and degradation (*33*, *34*). In both HCT116 and SW480 cells, β-catenin down regulation by siRNA, decreases β-TrCP protein (Fig 2A). To determine whether β-catenin protein affects the half-life of PER2 protein, we compared the half life of PER2 in control and β-catenin down-regulated cells. When protein translation is blocked by cycloheximide (CHX), PER2 protein is more stable in β-catenin down regulated cells (Fig 2B). On the other hand, co-expression of wild-type β-catenin or β-catenin S37A mutant markedly shortens PER2 protein half-life in 293T cells (Fig. 2C).

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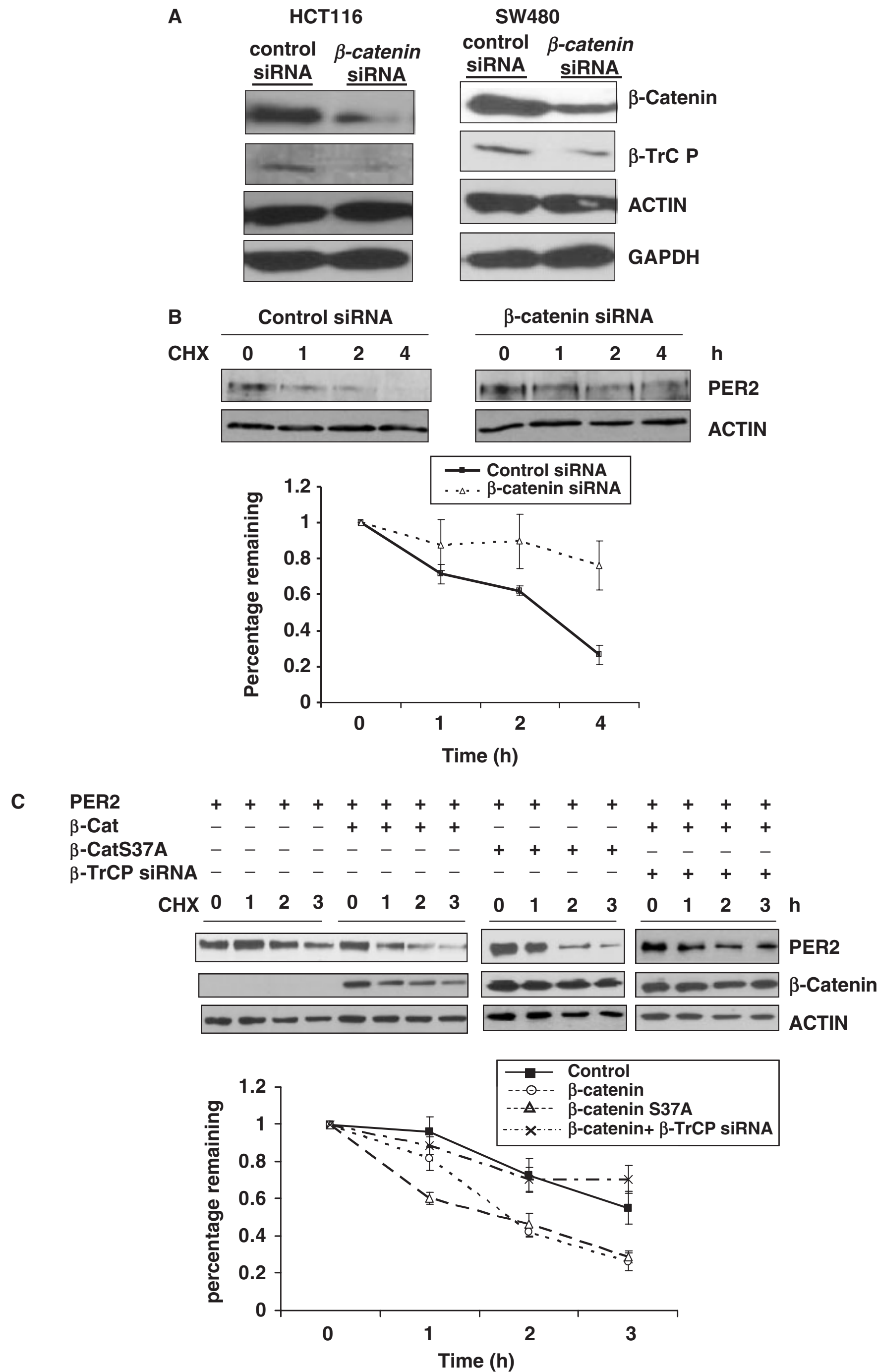

Fig. 2. **β-Catenin modulates PER2 protein stability**. (A) Immunoblot assay of β-catenin and β-TrCP protein levels with β-catenin knockdown (HCT116, left; SW480, right). (B) PER2 protein stability in β-catenin siRNA treated cells. HCT116 cells were treated with the control (left panel) or β-catenin siRNA (right panel). After CHX addition, total cell extracts were examined by immunobloting from samples taken at the indicated times. Low panel is the quantification of remaining PER2 protein of the control (solid line) and β-catenin down regulated cells (dashed line). (C) PER2 protein stability in β-catenin overexpressed cells. *Per2* was co-expressed with the vector, β-catenin, β-catenin S37A, or β-catenin and β-TrCP siRNA in 293T cells. PER2 protein levels were assayed after CHX treatment at the indicated times (top panels). The remaining PER2 protein was quantified (lower panel). Values are means and standard errors.

293T cells were chosen because of their high transfection efficiency, low endogenous β-catenin concentration, and because it is known that β-catenin significantly increases β-TrCP protein in this cell line (*33*, *34*). This β-catenin enhanced PER2 degradation is reversed when β-TrCP is down regulated by siRNA (Fig. 2C).

Altered PER2 Protein Circadian Rhythm and Levels in the Small Intestinal Mucosa of $Apc^{Min/+}$ Mice—Because

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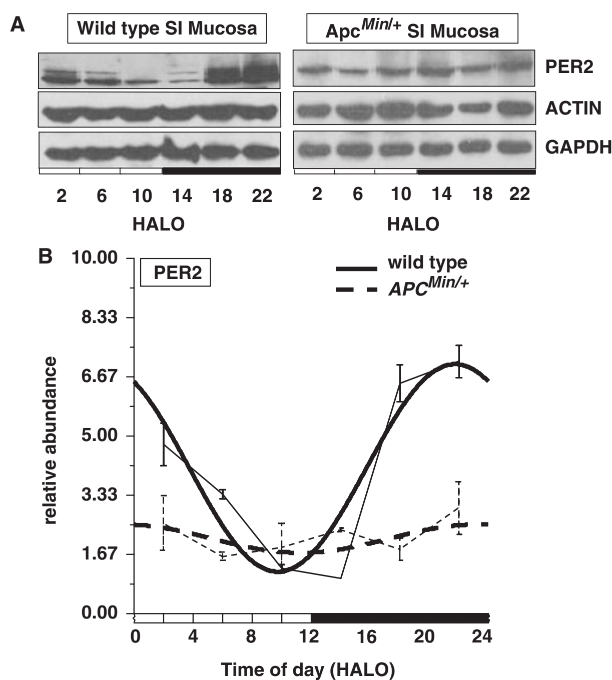

Fig. 3. **Daily levels and rhythm in PER2 protein expression in small intestinal mucosa in** $Apc^{Min/+}$ **mice are markedly depressed**. (A) PER2 protein from mucosa of wild-type and $Apc^{Min/+}$ mice at six different HALOs (time of day) by western blots. ACTIN and GAPDH serve as loading control (left panel). (B) Quantitation of PER2 levels (ratio to control protein) with mean ± SE in wild type (solid line) and $Apc^{Min/+}$ (dashed line) mice at different times of day with cosine fitted curve for 24 h period. Results are normalized to the lowest wild-type value (right panel). Values are means ± SE from six to eight mice/HALO.

β-catenin enhances the degradation of PER2 *in vitro*, we examined PER2 protein concentration in the intestinal mucosa of $Apc^{Min/+}$ mice. In wild-type intestinal mucosa, PER2 protein shows a 6–7-fold daily variation ($P < 0.001$) (Fig 3). In $Apc^{Min/+}$ mucosa, daily PER2 protein concentration rhythm is lost ($P = 0.51$) and PER2 protein levels are, overall, diminished up to 6-fold throughout of the 24 h cycle, compared to the rhythmic wild-type intestinal mucosa (Fig. 3).

Daily Expression Patterns of Core-Clock Genes and Clock-Controlled Genes in the Small Intestinal Mucosa of $Apc^{Min/+}$ *Mice*—Altered levels of the core circadian clock component, PER2 protein, can be expected to affect the circadian expression patterns of other clock genes, in addition to its own transcription. This should be reflected in alterations in other circadian clock gene and clock output gene expression patterns. We, therefore, contrasted the circadian clock gene expression profiles of intestinal mucosa in wild type and $Apc^{Min/+}$ mice. The peak daily expression of *Per1* in the intestinal epithelium of wild-type mice occurs near the sleep-wake interface (rhythm $P < 0.001$, acrophase: HALO 12.7 ± 0.8), a phase quite similar to that in liver, lung and heart (*8*, *35*). A circadian rhythm of *Per1* persists in $Apc^{Min/+}$ mouse intestinal mucosa (rhythm $P < 0.001$, acrophase: HALO 7.4 ± 0.8), but, its maximal expression is shifted to the middle of the daily sleep span (Fig. 4A). Although the timing of *Per1* peak is affected, neither the overall average daily level (mesor: wild type $= 2.76 \pm 0.25$ *vs.* $Apc^{Min/+} = 2.98 \pm 0.27$, P = ns) nor the amplitude of *Per1* expression are altered (wild type $= 3.5 \pm 0.8$ *vs.* $Apc^{Min/+} = 3.6 \pm 0.8$, P = ns). Although PER2 protein in $Apc^{Min/+}$ mucosa is non-rhythmic, *Per2* RNA in intestinal mucosa of $Apc^{Min/+}$ mice is still rhythmic ($P < 0.01$). The *Per2* RNA rhythm, however, is severely damped when compared with the daily *Per2* RNA rhythm in the wild-type intestinal epithelium (amplitude wild type $= 8.9 \pm 1.2$ *vs.* $Apc^{Min/+} = 3.2 \pm 0.6$, $P = 0.01$, Fig. 4B). Timing of the daily peak *Per2* gene expression is identical in the intestinal mucosa from mice of both strains (acrophase, wild type: HALO 12.8 ± 0.5 *vs.* $Apc^{Min/+}$:HALO 12.9 ± 0.8, P = ns).

The expression of another core clock gene, *Bmal1*, maintains almost identical circadian orientation (peak time, amplitude, mesor by cosinor analysis) in both wild type and $Apc^{Min/+}$ mouse intestinal epithelium (Fig 4C). The daily rhythm of *Rev-erbα*, which controls the rhythm of *Bmal1* expression, is also unaltered in $Apc^{Min/+}$ mucosa (Fig. 4D). The daily expression pattern of the clock output gene, *Dbp* (*36*, *37*), has a robust 200-fold circadian rhythm in wild-type small intestinal mucosa. *Dbp* maintains its usual circadian rhythm and phase but its daily variation is damped by nearly 4-fold in $Apc^{Min/+}$ mouse intestinal epithelium (amplitude, 66 ± 9.6 fold). Daily *Dbp* mean expression levels are also reduced (mesor, wild type $= 63.4 \pm 7.3$ *vs.* $Apc^{Min/+} = 25.3 \pm 3.4$, $P < 0.001$, Fig 4E). *Wee1*, an important circadian clock controlled cell cycle regulator, which blocks cells from entering mitosis, has a robust circadian oscillation in the intestinal mucosa of wild-type mice, peaking in the early active (dark) phase. *Wee1* RNA rhythm is, however, much damped (amplitude, wild type $= 5.4 \pm 0.8$ *vs.* $Apc^{Min/+} = 1.6 \pm 0.4$) and overall levels (mesor, wild type $= 5.7 \pm 0.3$ *vs.* $Apc^{Min/+} = 1.8 \pm 0.1$) are also diminished nearly 3-fold, throughout the day, in intestinal epithelial from $Apc^{Min/+}$ mice (Fig 4F).

$Apc^{Min/+}$ *Mice Have Normal Circadian Locomotor Activity*—$Apc^{Min/+}$ mice develop intestinal and colonic polyps and eventually lose weight and die at 4–5 months of age. However, 15-week-old female $Apc^{Min/+}$ mice, do not yet exhibit differences in body weight compared to wild-type mice (20.7 ± 0.2 g *vs.* 20.3 ± 0.6 g, $P = 0.45$). To rule out the possibility that $Apc^{Min/+}$ mice might have an abnormality in their central clock, we compared circadian locomotor activities of wild type and $Apc^{Min/+}$ mice. In 12 h light 12 h dark condition (LD), $Apc^{Min/+}$ mice show similar overall wheel running activity levels and rhythms (Table 2). The 24 h mean and amplitude of locomotor rhythms in $Apc^{Min/+}$ and wild-type mice are identical. In constant darkness (DD), wild type and $Apc^{Min/+}$ mice both free run with identical endogenous period lengths (Tau; 23.5 ± 0.04 *vs.* 23.6 ± 0.05 h in $Apc^{Min/+}$ and wild-type mice, respectively). Moreover, in constant darkness, day-to-day variability is not altered, as indicated by unchanged interdaily stability and 24 h autocorrelation of the daily rhythms in $Apc^{Min/+}$ and wild-type mice (Table 2). These results indicate that $Apc^{Min/+}$ mice have a normal master clock residing in their suprachiasmatic nuclei (SCN) and that the effects

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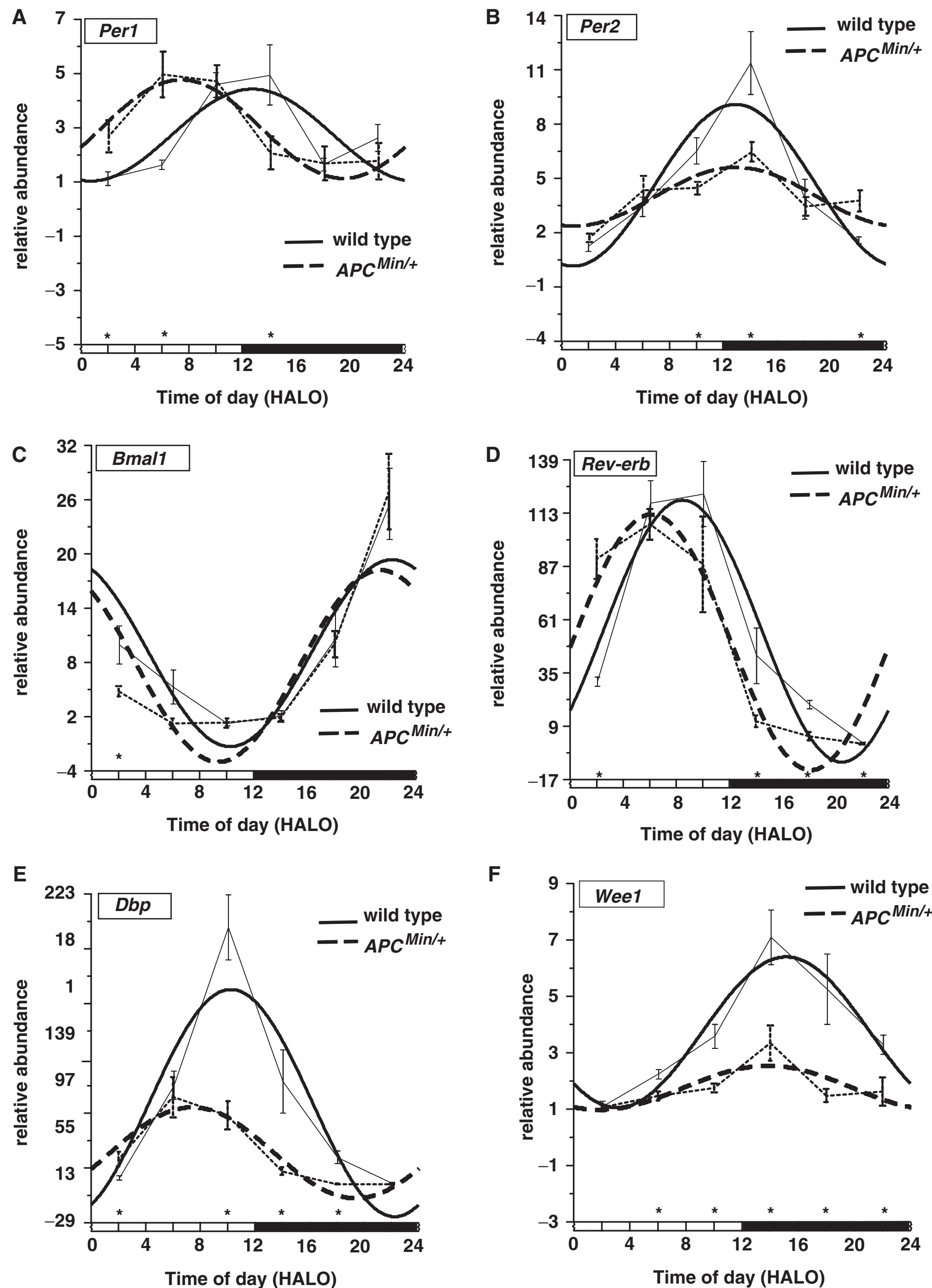

Fig. 4. **Rhythmic expression of clock genes in the small intestinal mucosa throughout the day.** Small intestinal mucosa of the wild-type (solid line) and $Apc^{min/+}$ mice (dash line) was collected at six times a day with seven to eight mice per time point. Relative RNA amounts of *Per1* (A), *Per2* (B), *Bmal1* (C), *Rev-erba* (D), *DbP* (E) and *Wee1* (F) were quantified by real time PCR using *Gapdh* as the internal control. The lowest levels of the wild type were set as 1. Cosine fit curves (24 h) are shown along with mean and standard errors of data (*significant difference between wild type and $Apc^{Min/+}$ at that time of day, $P < 0.05$).

we observe in the peripheral clock in the intestinal epithelium are tissue specific.

DISCUSSION

β-Catenin/TCF signaling is increased in colon cancers. β-catenin/TCF transcriptional complex activates the transcription of an RNA-binding protein, CRD-BP, which in turn stabilizes β-TrCP mRNA and leads to the elevated β-TrCP protein (*33*). In human colon cancer cells, we show that down regulation of β-catenin by siRNA decreases β-TrCP protein level, which in turn diminishes PER2 clock protein degradation and increases its half-life. The availability of ubiquitin E3 ligases is the rate-limiting step controlling the stability of such targeted proteins. The $SCF^{\beta\text{-TrCP}}$ ubiquitin E3 ligase recognizes and polyubiquitinates phosphorylated PER2 protein and triggers its proteasome-mediated degradation.

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Table 2. **Circadian locomotor activity parameters of $Apc^{Min/+}$ and wild-type mice in LD and DD lighting conditions**

Light condition	Activity parameters	Strain		Strain difference
		$Apc^{Min/+}$	Wild type	(*F*, *P*)
12L/12D	24 h mean	496.1 ± 112.0	431.3 ± 105.0	0.1, 0.90
	Amplitude	730.0 ± 163.0	646.9 ± 155.0	0.1, 0.90
	Interdaily Stability (IS)[a]	0.72 ± 0.07	0.57 ± 0.09	1.71, 0.19
	Interdaily Variablility (IV)[b]	0.57 ± 0.12	0.70 ± 0.15	0.02, 0.87
	24 h autocorrelation[c]	0.67 ± 0.07	0.60 ± 0.07	0.03, 0.87
D/D	24 h mean	508.8 ± 102.0	518.8 ± 115.0	0.1, 0.90
	Amplitude	722.0 ± 156.0	730.0 ± 130.0	0.1, 0.90
	Tau[d]	23.50 ± 0.04	23.60 ± 0.05	0.9, 0.30
	Interdaily Stability (IS)	0.54 ± 0.08	0.53 ± 0.10	0.24, 0.62
	Interdaily Variablility (IV)	0.45 ± 0.46	0.75 ± 0.20	0.1, 0.90
	24 h autocorrelation	0.60 ± 0.06	0.53 ± 0.10	0.1,0.90

[a]IS, pattern stability. [b]IV, defragmentation of sleep. [c]24 h autocorrelation, pattern similarity between two 24 h periods. [d]Tau, circadian period (h); difference between when activity starts between days.

We show in the intestinal epithelium of $Apc^{Min/+}$ mice, the expression of PER2 protein is markedly decreased and its circadian rhythm is lost. Most studies indicate that β-catenin distribution and level in normal appearing intestinal mucosa of $Apc^{Min/+}$ mice is relatively normal compared with polyps. However, a couple of studies indicate that in the normal appearing mucosa of $Apc^{Min/+}$ mice, β-catenin protein is elevated compared with tissues from wild-type mice (*38*, *39*). Examining β-catenin and β-TrCP expression in $Apc^{Min/+}$ mucosa is necessary to define the mechanism. It is also possible that mechanisms other than enhanced β-TrCP contribute to decreased PER2 in $Apc^{Min/+}$ mucosa. In polyps from $Apc^{Min/+}$ mice and colorectal cancers, the β-catenin protein level is much higher than in non-polyp bearing mucosa (*26–28*). This may lead to elevated β-TrCP (*33*, *34*). We thereby expect the levels of PER2 protein might even be lower in polyps. In our studies, all visible polyps have been dissected from the intestinal mucosa of $Apc^{Min/+}$. It is possible that small polyps still remain in these mucosa samples. This technical limitation may, in part, contribute to the difference of PER2 protein expression we observed in $Apc^{Min/+}$ mucosa compared to wild type.

This β-catenin-mediated β-TrCP effect is also likely to affect PER1 protein stability (*11*). Deregulation of PER2, however, has more profound effects upon circadian timekeeping than the deregulation of PER1 expression. For example, circadian mRNA rhythms of *Per2*, *Bmal1* and *Cry1* do not change within the SCN of *Per1* mutant mice. But the rhythms of all these genes are damped in the SCN from *Per2* mutant mice (*40*).

The circadian expression of key clock genes, *Per1* and *Per2*, in intestinal mucosa of $Apc^{Min/+}$ mice is disordered compared to wild type. A decrease in PER2 protein levels, because of its negative feed back on its own transcription, will result in a decrease in *Per2* RNA levels. This is exactly what we observe in $Apc^{Min/+}$ mucosa with marked depression of PER2 levels throughout the day and a profound damping in the daily *Per2* RNA rhythm. In *Per2* mutant mice, the timing of the daily peak in liver *Per1* RNA is shifted 4 h earlier (*19*). This is exactly what we see in $Apc^{Min/+}$ mucosa, where PER2 levels are also greatly decreased, but by a different mechanism. Deregulation of *Period* gene expression necessarily disrupts the clock (*7*, *40*, *41*). Clock disruption in $Apc^{Min/+}$ mucosa is heralded by a circadian clock controlled output gene, *Dbp*, suppression and damping compared to wild type. Circadian clock function depends much more on *Period* (the negative feed back loop) than on *Bmal1* (the positive feed back loop) (*7*, *40*, *41*). This is consistent with our findings that the daily expression patterns of intestinal *Bmal1* and *Rev-erbα* clock genes, the positive loop of the clock are not affected by the $Apc^{Min/+}$ mutation.

The transcription of *Wee1*, a mitosis inhibitor and clock output gene, is activated by CLOCK/BMAL1 and inhibited by PER/CRY through E-box interactions (*16*). *Wee1* expression in $Apc^{Min/+}$ is suppressed and the daily rhythm flattened compared with the rhythm in the wild-type intestinal epithelium. It will be important to determine whether the clock-controlled activity of other cell cycle regulators and the daily timing of S phase and mitosis entry and exit differ in intestinal mucosa of wild type and $Apc^{Min/+}$ mice. The daily rhythms of epithelial cell proliferation, in the rectal mucosa of patients with colorectal cancer appear to be disturbed, compared with non-cancer bearing individuals (*42*, *43*).

In summary, it appears that the constitutive expression of β-catenin affects circadian clock, in part, through β-TrCP-mediated PER2 protein degradation. Since β-TrCP has been shown to be over-expressed in human colorectal cancer, this may be the underlying basis for altered *Per2* levels in colorectal cancer. Our current work suggests that deregulation of peripheral circadian clock may, in part, contribute to intestinal epithelial tumorigenesis in $Apc^{Min/+}$ mice and perhaps, human colorectal cancer. Determining how a disrupted circadian peripheral clock contributes to intestinal epithelial neoplastic transformation and how to prevent this disruption opens a novel circadian clock-based strategy to prevent and control colorectal cancer.

ACKNOWLEDGEMENTS

We thank Steven Reppert and David Weaver for *Per2*-V5 plasmid, Shi Lei for β-catenin expression plasmid, Tia Davis for animal breeding and Dinah Quiton for help with manuscript preparation.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. You, S., Wood, P.A., Xiong, Y., Kobayashi, M., Du-Quiton, J., and Hrushesky, W.J. (2005) Daily Coordination Of Cancer Growth And Circadian Clock Gene Expression. *Breast Cancer Res. Treat.* **91**, 47–60
2. Wood, P.A., Du-Quiton, J., You, S., and Hrushesky, W.J. (2006) Circadian clock coordinates cancer cell cycle progression, thymidylate synthase, and 5-fluorouracil therapeutic index. *Mol. Cancer Ther.* **5**, 2023–2033
3. Klevecz, R.R., Shymko, R.M., Blumenfeld, D., and Braly, P.S. (1987) Circadian gating of S phase in human ovarian cancer. *Cancer Res.* **47**, 6267–6271
4. Hrushesky, W.J. (1985) Circadian timing of cancer chemotherapy. *Science* **228**, 73–75
5. Wood, P.A., Hrushesky, W.J., and Klevecz, R. (1998) Distinct circadian time structures characterize myeloid and erythroid progenitor and multipotential cell clonogenicity as well as marrow precursor proliferation dynamics. *Exp Hematol.* **26**, 523–533
6. Hrushesky, W.J.M., Lannin, D., and Haus, E. (1998) Evidence for an ontogenetic basis for circadian coordination of cancer cell proliferation. *J. Nat. Cancer Inst.* **90**, 1480–1484
7. Reppert, S.M. and Weaver, D.R. (2002) Coordination of circadian timing in mammals. *Nature* **418**, 935–941
8. Lee, C., Etchegaray, J.P., Cagampang, F.R., Loudon, A.S., and Reppert, S.M. (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell.* **107**, 855–867
9. Yamamoto, Y., Yagita, K., and Okamura, H. (2005) Role of cyclic mPer2 expression in the mammalian cellular clock. *Mol. Cell Biol.* **25**, 1912–1921
10. Gallego, M. and Virshup, D.M. (2007) Post-translational modifications regulate the ticking of the circadian clock. *Nat. Rev. Mol. Cell Biol.* **8**, 139–148
11. Shirogane, T., Jin, J., Ang, X.L., and Harper, J.W. (2005) SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *J. Biol. Chem.* **280**, 26863–26872
12. Eide, E.J., Woolf, M.F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E.L., Giovanni, A., and Virshup, D.M. (2005) Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol. Cell Biol.* **25**, 2795–2807
13. Siepka, S.M., Yoo, S.H., Park, J., Song, W., Kumar, V., Hu, Y., Lee, C., and Takahashi, J.S. (2007) Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* **129**, 1011–1023
14. Miller, B.H., McDearmon, E.L., Panda, S., Hayes, K.R., Zhang, J., Andrews, J.L., Antoch, M.P., Walker, J.R., Esser, K.A., Hogenesch, J.B., and Takahashi, J.S. (2007) Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc. Natl Acad. Sci. USA* **104**, 3342–3347
15. Koyanagi, S., Kuramoto, Y., Nakagawa, H., Aramaki, H., Ohdo, S., Soeda, S., and Shimono, H. (2003) A molecular mechanism regulating circadian expression of vascular endothelial growth factor in tumor cells. *Cancer Res.* **63**, 7277–7283
16. Matsuo, T., Yamaguchi, S., Mitsui, S., Emi, A., Shimoda, F., and Okamura, H. (2003) Control mechanism of the circadian clock for timing of cell division *in vivo*. *Science* **302**, 255–259
17. Griniatsos, J., Michail, O.P., Theocharis, S., Arvelakis, A., Papaconstantinou, I., Felekouras, E., Pikoulis, E., Karavokyros, I., Bakoyiannis, C., Marinos, G., Bramis, J., and Michail, P.O. (2006) Circadian variation in expression of G1 phase cyclins D1 and E and cyclin-dependent kinase inhibitors p16 and p21 in human bowel mucosa. *World J. Gastroenterol.* **12**, 2109–2114
18. Fu, L. and Lee, C.C. (2003) The circadian clock: pacemaker and tumour suppressor. *Nat. Rev. Cancer* **3**, 350–361
19. Fu, L., Pelicano, H., Liu, J., Huang, P., and Lee, C. (2002) The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**, 41–50
20. Chen, S.T., Choo, K.B., Hou, M.F., Yeh, K.T., Kuo, S.J., and Chang, J.G. (2005) Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. *Carcinogenesis* **26**, 1241–1246
21. Sjoblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S.D., Willis, J., Dawson, D., Willson, J.K., Gazdar, A.F., Hartigan, J., Wu, L., Liu, C., Parmigiani, G., Park, B.H., Bachman, K.E., Papadopoulos, N., Vogelstein, B., Kinzler, K.W., and Velculescu, V.E. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268–274
22. Zeman, M., Vician, M., Monosikova, J., Reis, R., and Herichova, I. (2008) Deregulated expression of the Per2 gene in human colorectal carcinoma. *Molecular Med. Rep.* **1**, 599–603
23. Yang, X., Wood, P.A., Oh, E.Y., Du-Quiton, J., Ansell, C.M., and Hrushesky, W.J. (2008) Down regulation of circadian clock gene Period 2 accelerates breast cancer growth by altering its daily growth rhythm. *Breast Cancer Res. Treat*.
24. Wood, P.A. and Hrushesky, W.J. (1996) Circadian rhythms and cancer chemotherapy. *Crit. Rev. Eukaryot. Gene Expr.* **6**, 299–343
25. Wood, P.A., Yang, X., Taber, A., Oh, E.Y., Ansell, C., Ayers, S.E., Al-Assaad, Z., Carnevale, K., Berger, F.G., Peña, M.M.O., and Hrushesky, W.J.M. (2008) *Period 2* Mutation Accelerates $Apc^{Min/+}$ Tumorigenesis. *Mol. Can. Res.* **6**, 1786–1793
26. Karim, R., Tse, G., Putti, T., Scolyer, R., and Lee, S. (2004) The significance of the Wnt pathway in the pathology of human cancers. *Pathology* **36**, 120–128
27. Kolligs, F.T., Bommer, G., and Goke, B. (2002) Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion* **66**, 131–144
28. Schneikert, J. and Behrens, J. (2007) The canonical Wnt signalling pathway and its APC partner in colon cancer development. *Gut.* **56**, 417–425
29. Frescas, D. and Pagano, M. (2008) Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat. Rev. Cancer.* **8**, 438–449
30. Ougolkov, A., Zhang, B., Yamashita, K., Bilim, V., Mai, M., Fuchs, S.Y., and Minamoto, T. (2004) Associations among beta-TrCP, an E3 ubiquitin ligase receptor, beta-catenin, and NF-kappaB in colorectal cancer. *J. Natl. Cancer Inst.* **96**, 1161–1170
31. Lincoln, D.W., II, Hrushesky, W.J., and Wood, P.A. (2000) Circadian organization of thymidylate synthase activity in normal tissues: a possible basis for 5-fluorouracil chronotherapeutic advantage. *Int. J. Cancer.* **88**, 479–485
32. He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509–1512
33. Noubissi, F.K., Elcheva, I., Bhatia, N., Shakoori, A., Ougolkov, A., Liu, J., Minamoto, T., Ross, J., Fuchs, S.Y., and Spiegelman, V.S. (2006) CRD-BP mediates stabilization of betaTrCP1 and c-myc mRNA in response to beta-catenin signalling. *Nature* **441**, 898–901

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34. Spiegelman, V.S., Slaga, T.J., Pagano, M., Minamoto, T., Ronai, Z., and Fuchs, S.Y. (2000) Wnt/beta-catenin signaling induces the expression and activity of betaTrCP ubiquitin ligase receptor. *Mol Cell* **5**, 877–882
35. Yamamoto, T., Nakahata, Y., Soma, H., Akashi, M., Mamine, T., and Takumi, T. (2004) Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Mol. Biol.* **5**, 18
36. Ripperger, J.A. and Schibler, U. (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat. Genet.* **38**, 369–374
37. Ripperger, J.A., Shearman, L.P., Reppert, S.M., and Schibler, U. (2000) CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev.* **14**, 679–689
38. Ghaleb, A.M., McConnell, B.B., Nandan, M.O., Katz, J.P., Kaestner, K.H., and Yang, V.W. (2007) Haploinsufficiency of Kruppel-like factor 4 promotes adenomatous polyposis coli dependent intestinal tumorigenesis. *Cancer Res.* **67**, 7147–7154
39. Mahmoud, N.N., Boolbol, S.K., Bilinski, R.T., Martucci, C., Chadburn, A., and Bertagnolli, M.M. (1997) Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. *Cancer Res.* **57**, 5045–5050
40. Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., and Weaver, D.R. (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron* **30**, 525–536
41. Albrecht, U., Zheng, B., Larkin, D., Sun, Z.S., and Lee, C.C. (2001) MPer1 and mper2 are essential for normal resetting of the circadian clock. *J. Biol. Rhythms* **16**, 100–104
42. Biasco, G., Cenacchi, G., Nobili, E., Pantaleo, M.A., Calabrese, C., Di Febo, G., Morselli Labate, A., Miglioli, M., and Brandi, G. (2004) Cell proliferation and ultrastructural changes of the duodenal mucosa of patients affected by familial adenomatous polyposis. *Hum. Pathol.* **35**, 622–626
43. Brandi, G., Calabrese, C., Pantaleo, M.A., Morselli Labate, A., Di Febo, G., Hakim, R., De Vivo, A., Di Marco, M.C., and Biasco, G. (2004) Circadian variations of rectal cell proliferation in patients affected by advanced colorectal cancer. *Cancer Lett.* **208**, 193–196

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