Articles in PresS. Am J Physiol Regul Integr Comp Physiol (October 5, 2016). doi:10.1152/ajpregu.00032.2016

Title: CLOCK Regulates Mammary Epithelial Cell Growth and Differentiation

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Running Head: CLOCK Regulates Mammary Growth and Differentiation

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ABSTRACT

Circadian clocks influence virtually all physiological processes, including lactation. Here we investigate the role of the CLOCK gene in regulation of mammary epithelial cell growth and differentiation. Comparison of mammary morphology in late pregnant wild-type and *ClockΔ19* mice*,* showed that gland development was negatively impacted by genetic loss of a functional timing system. To understand if these effects were due in part to loss of CLOCK function in the gland, the mouse mammary epithelial cell line, HC11, was transfected with shRNA that targeted *Clock* (shClock). Cells transfected with shClock expressed 70% less *Clock* mRNA than wild-type (WT) HC11 cultures, which resulted in significantly depressed levels of CLOCK protein ($P < 0.05$). HC11 lines carrying shClock had 4-fold higher growth rates ($P < 0.05$), and percent of cells in G1 phase was significantly higher (90.1± 1.1% of shClock versus 71.3 ± 3.6% of WT-HC11) following serum starvation. Q-PCR analysis showed shClock had significant effects ($P < 0.0001$) on relative expression levels of *Ccnd1*, *Wee1* and *Tp63.* Q-PCR analysis of effect of shClock on *Fasn* and *Cdh1* expression in undifferentiated cultures, and cultures treated 96 h with dexamethasone, insulin and prolactin (differentiated), found levels were reduced by 2-fold and 3-fold, respectively ($P < 0.05$), in sh*Clock* line relative to WT cultures. Abundance of CDH1 and TP63 proteins were significantly reduced in cultures transfected with sh*Clock*. These data support CLOCK plays a role in regulation of epithelial cell growth and differentiation in the mammary gland.

Key words. circadian, CLOCK, lactation, mammary development

INTRODUCTION

Dynamic changes occur in molecular clocks across multiple tissues during the transition from pregnancy to lactation. Amplitude and robustness of rhythms of clock genes in SCN and liver are increased. In the mammary gland the stoichiometric relationship of core clock proteins change, with ratio of BMAL1 and CLOCK increasing relative to a decrease in PER2 levels during the transition from pregnancy to lactation (5). *In vitro* studies demonstrated that these changes in mammary core clock dynamics were associated with development. Moreover, temporal expression patterns of core clock genes change throughout mammary development, and these changes were coincident with changes in peak of proliferation (pregnancy) and differentiation (lactation) state of the gland (23). The significance of these changes and the role of molecular clocks, in particular the mammary clock, in regulation of lactation competence is currently not understood.

Studies of circadian regulation of reproduction in the *Clock-Δ19* line of mice found this mutation has minimal effects on growth and development of pups during gestation, but litter growth and survival is significantly decreased postnatally (7, 14, 15). The increased pup mortality rate is evident in both heterozygous and homozygous *Clock-Δ19* mutant offspring; thus increased pup mortality is likely due to a maternal defect in circadian clocks that causes a decrease in lactation competency in this line of mice. *Clock-Δ19* mice have an ENU-induced mutation that affects transactivation properties of CLOCK, and results in disruption of behavioral rhythmicity, loss of rhythmic gene expression, and down-regulation of CLOCK-BMAL1 target genes (2, 16, 26). Thus in the *Clock-Δ19* mutant line, circadian clocks are compromised in both central and peripheral tissues. Dolastad et al. (2006) compared *Clock-Δ19* to V*ipr2*$^{-/-}$ lines of mice to understand if impact on litter growth and survival was due to loss of circadian clock function centrally versus across the whole animal, as mice with a null mutation of the VPAC2 receptor gene (Vipr2–/–) only have a deficient clock in the master clock in the suprachiasmatic nuclei-SCN (13), while peripheral tissue molecular clocks are not directly affected. They found pups born to *Clock-Δ19* dams had significantly reduced survival to weaning compared to pups born to wild-type or *Vipr2*$^{-/-}$ dams, suggesting that loss of peripheral clock function was impairing lactation competence in the *Clock-*

Δ19 line. Lactation competency can be affected by multiple interacting factors spanning from maternal behavior to systemic hormones to mammary development. Hoshino et al. (2006) reported altered maternal nursing behavior in the *Clock-Δ19* line of mice. Specifically, they found that the daily rhythm of maternal nursing behavior had a strong diurnal peak and two weak nocturnal peaks in wild-type dams, whereas *Clock-Δ19* dams exhibited no significant peaks in activity(14). Moreover, the duration of nursing bouts was significantly longer in wild-type mice versus Clock mutants. However, the number of nursing events per day was greater in *Clock-Δ19* line versus wild-type animals. Hoshino et al. (2006) also found wild-type, but not *Clock-Δ19,* dams showed circadian rhythms of prolactin serum content, however there was no significant difference in mean daily prolactin levels between the lines. Thus, although maternal behavior and systemic hormones are impacted by *Clock-Δ19* mutation, it is not likely that mutation effects on nursing behavior and prolactin can fully explain the poorer lactation competence in this line of mice.

Endogenous clocks generate circadian rhythms through a series of interlocked transcription-translation feedback loops. At the core of the loops are two transcription factors, CLOCK (or its paralog NPAS2) and BMAL1. CLOCK-BMAL1 heterodimers bind the E-box regulatory element in promoter regions of genes (12), including *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. PER and CRY proteins function as negative regulators of CLOCK-BMAL1-mediated transcription (1, 25, 31). The 24 h periodicity in activation-repression of the molecular clock results in circadian oscillation in approximately 10% of mammalian transcriptome, which translates into daily variations in cellular, metabolic and physiological functions (25). Rhythmic output genes differ among tissues, allowing circadian control of function and activity appropriate for each organ. In the mammary, seven percent of genes expressed in the gland during lactation exhibit circadian oscillation, and these genes function to regulate cell proliferation and differentiation, as well as metabolic output (20). Cell cycle regulators and tumor suppressors have been identified as clock controlled genes in multiple tissues (9, 25, 28), and circadian control of these genes is believed to be important to the coupling of regulation of proliferation with key tissue functions. Thus it is likely that circadian clocks play a similar role in the mammary gland. In addition our previous studies of cattle

showed that exposure to chronic shifts of light-dark cycle, which disrupts the circadian system, decreased milk production and impacted genes important to milk synthesis(4), suggesting that clocks play an important role in regulation of milk synthesis.

We hypothesize that the circadian clock in the mammary gland plays an integral role in regulating mammary gland development as well as metabolic output during lactation, and thus mutation or decreased expression of the CLOCK gene in mammary epithelial cells will impact growth and differentiation. Here we show that in late pregnancy morphological development of the mammary gland was impaired in *Clock-Δ19* mice compared to wild-type dams. Poorer development of the gland was associated with lower survival rates of pups born to *Clock-Δ19* dams. To determine whether loss of CLOCK function in mammary epithelial cells could account for this phenotype, we used a mouse mammary epithelial cell line, HC11, to study the impact of decreasing CLOCK levels with shRNA on growth and differentiation of cultures. HC11 cells undergo differentiation upon lactogen treatment, and our previous studies demonstrated periparturient changes in mammary clock dynamics can be mimicked in culture upon treatment with prolactin and glucocorticoids (5). Here we report that reducing CLOCK levels in HC11 cells, increased growth rates and decreased expression of factors associated with mammary differentiation and metabolic output.

MATERIAL AND METHODS

***Animal Studies*.** All animal work was performed in the Roswell Park Cancer Institute (RPCI) vivarium with IACUC approval. C57Bl6/J wild type (WT) male and female mice were purchased from the Jackson Laboratories, and maintained on 12h light: 12h dark light dark cycle with ad libitum access to food and water. The breeding colony of *Clock*-Δ19 mice was maintained in RPCI vivarium. Female WT and *Clock*-Δ19 mice were mated on a 1:1, female: male ratio with WT males, and checked daily for vaginal plugs (pregnancy day 1).

Mice were anesthetized with 5% isoflurane with 1 l/min O_2 on pregnancy day 16-17 blood and abdominal mammary glands were collected at 6 and 10 h (n=3/genotype/time point) after lights on. Blood was collected, plasma prepared and

stored at -20°C until analysis. Commercially available enzyme immunoassay (EIA) kits were used to measure plasma prolactin (Calbiotech, Spring Valley, CA) and corticosterone (ALPCO, Salem, NH) levels, following manufacturer's direction. Mammary no. 4 glands were removed and fixed in 10% buffered formalin for histological analysis. Number of fetuses were counted and recorded.

Histological analysis of mammary morphology. After overnight fixation in buffered formalin mammary tissue was transferred to phosphate buffered saline. Tissues were paraffin embedded and 7 µm sections were cut and placed on electrostatically charged slides. Hematoxylin and eosin (H&E) stained sections were used to evaluate the impact of *Clock*Δ19 mutation on mammary development. Slides were blinded by treatment, and to account for potential heterogeneity of tissue, three images of each H&E section were captured using a digital camera attached to a light microscope. The three areas were selected by first focusing scope at approximate center of tissue section, and then field of view was moved to 12, 4 and 8 o'clock relative to center and image was captured. Image Pro Plus software was used to measure luminal area and area of alveolar epithelium in three images with n=6 animals/treatment. Ratio of luminal area to alveolar epithelium was calculated, and expressed as mean ± standard error (SE).

Cell culture.

HC-11 cells were routinely cultured in complete growth medium (RPMI 1640 supplemented with 2g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% heat inactivated calf serum, 5 µg/mL insulin (Ins), and 10 ng/mL epidermal growth factor [EGF]) in 5% CO_2 at 37°C. Cells were passaged 1:3 when they reached 80% confluence by harvesting cells with 0.25% trypsin EDTA.

Four unique Qiagen SureSilencing shRNA plasmid sequences specific to the mouse *Clock* gene (Table 1) were transfected into HC-11 cells. Each shRNA plasmid carried a U1 promoter, a shRNA sequence, and hygromycin resistance gene. Plasmids were amplified in *E. coli* and isolated f following manufacturer's protocol (Qiagen Endofree Plasmid Maxi kit), and linearized with restriction enzymes (New England BioLabs, Inc.). For transfection, HC-11 cells were plated at a density of 100,000 cells/mL in 24-well cell culture plates with complete growth media. Linearized *Clock* shRNA plasmids were transfected into HC-11 cells using the lipofection Attractene

Transfection Reagent (Qiagen cat. no. 1051561). After transfected cells reached confluence, hygromycin was added at 200 µg/mL. Cells that were viable after 7 days of hygromycin selection, were used to create clonal lines by dilutional cloning, and the effect of abundance of *Clock* mRNA level was screened using q-PCR. Nontransfected HC-11 cells served as wild-type control.

For growth curve analysis, 100,000 cells were plated using complete growth media in 6-well dishes, and cells from 2-dishes per time point were harvested using trypsin EDTA and counted on days 2, 4 and 7 using a BioRad TC10 Automated cell counter. Growth curves were conducted 4-times, and results are presented as mean number of cells each day ± standard error (SE). Doubling time was calculated using the tool available here (33).

For cell cycle analysis, cells were plated overnight at a density of 100,000 cells/ml in RPMI media supplemented with Ins and 10% calf serum. Media was removed and cells were washed 2-times with PBS, and serum starved in RPMI media + Ins. After 24 h in serum free media, complete growth media [RPMI with 10% calf serum, Ins and EGF] was added (time 0 h), and cells were collected every 4 h over a 48 h period. At each time point cells were collected for cell cycle and Q-PCR analysis of gene expression. To label cells with propidium iodide (PI) for flow analysis of cell cycle phase, cell pellets of 1.5 $x10^6$ cells were resuspended in 100 µl of PBS and fixed with 280 µl of ice-cold 90% ethanol. Fixed cells were stored at -20° C until day of analysis. On day of analysis, fixed cells were pelleted by centrifugation and resuspended in 1ml PBS-0.5% BSA, pelleted again, resuspended in PBS-0.5% BSA + 100U/ml RNAse (R6513 Sigma-Aldrich), and incubated for 15 minutes at 37°C. Five µl of PI (500ug/ml stock solution) was added and cells were incubated 15 minutes at RT. PI labeled cells were analyzed by fluorescently activated cell sorting (FACS) using a Beckman Coulter FC500 instrument. FACS analysis was repeated 3-times.

To induce HC11 differentiation, cells were plated at 100,000 cells/mL and grown to confluence. At confluence cells were washed 2-times with PBS, and incubated for 48 h in RPMI media supplemented with 10% calf serum and Ins. Undifferentiated cultures were collected for isolation of RNA and protein after 48 h. For differentiated cultures, media was changed to RPMI supplemented with 10% calf serum, dexamethasone

(0.1uM), insulin (5ug/mL), and prolactin (5ug/mL) (DIP treated), and incubated for 96 h, with media change every 2-days. After 96 h in DIP, differentiated cultures were collected for RNA and protein isolation.

Protein Isolation and Western Blot Analysis

Protein lysates were isolated from cultures by pouring off media, washing cells two times with chilled PBS, and harvested using a scraper and 3 ml of cold PBS. Cells were pelleted by centrifugation, and cell pellets were lysed for 30 min on ice with 600 µl of Cell Extraction Buffer (Invitrogen, supplemented with 1mM of PMSF and 50 µl/ml of Protease Inhibitor Cocktail), with vortexing at 10 min intervals. Protein lysates were transferred to a microcentrifuge tube and centrifuged at 13,000rpm for 10 min at 4ºC. Protein was then aliquoted into microfuge tubes and stored at -80°C until further analysis. Protein concentration was measured using the Quick Start Bradford Protein Assay (BioRad).

Fifty µg of protein was loaded per lane and electrophoresed on a 10% TGX precast SDS PAGE gel from BioRad. Protein was transferred onto a Polyvinylidene fluoride (PVDF) membranes for western blot analysis. Membranes were probed for CLOCK, BMAL1, PER2, e-cadhein-CDH1, TP63 and B-ACTIN proteins using Anti-CLOCK (AbCam, ab3517, dilution 1:5000), anti-CDH1 (Cell Signaling, 24E10, dilution 1:1000) anti TP63 (Millipore, ABS552, dilution 1:7500) and Anti-B-ACTIN (AbCam ab8227, dilution 1:7500) antibodies, respectively. The secondary antibody (ab97051) was then incubated on the blots at the dilution of 1/10,000. Membranes were washed with the detection reagent Clarity Western ECL Substrate (BioRad). Blots were imaged using the ChemiDoc MP system (BioRad), and the relative band intensity was measured using Image J software. Relative protein abundance was determined by calculating ratio of target protein to beta actin, and then normalized relative to WT HC11 cultures by dividing by ratio value. To accommodate multiple treatments there were times two gels needed to be run. When this was done gels were loaded, run and transferred to membranes simultaneously. Followed by membrane incubation with antibody probes in same solutions, to allow for band intensities to be compared across blots. Data are mean ± S.E. of relative ratios across at least 3 experiments.

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232 ***RNA Isolation and Real-Time Quantitative PCR Analysis (RT-qPCR)***

233 RNA was collected from cells and isolated using Qiagen's RNeasy kit, and quantity was
234 measured using Nanodrop. Gene expression was analyzed using TaqMan One-Step
235 RT-qPCR (Life Technologies). Multiple genes (beta-actin, beta microglobulin and 18S)
236 were analyzed to serve as reference (housekeeping gene) for calculating relative
237 expression using the $2^{-\Delta\Delta CT}$ method (19);18S was chosen as the reference gene based
238 on its levels staying steady across time and genotype. Commercially available (Life
239 Technologies) mouse-specific TaqMan assays were used to measure the expression of
240 *Clock, Fasn, TP63, Wee1, Csn2, Cdh1, Ccnd1* and *18S*.

241 To calculate relative expression, mean ΔCT of wild-type cultures across all time
242 points for cell cycle analysis or mean ΔCT of undifferentiated cultures was used as
243 normalizer for relative expression. Except where noted, data are presented as mean
244 log base 2-fold change ± SE of relative expression levels.

245 ***Data and Statistical Analysis***

246 Statistical analysis was performed using Minitab software. ANOVA was used to analyze
247 whether shRNA sequences significantly impacted variables (relative expression, protein
248 abundance, doubling time, and cell density) relative to wild-type HC-11 cultures.
249 General linear model was used to determine the effect of treatment and time on cell
250 number. A Tukey's test was used for post hoc pairwise comparisons. Significance was
251 considered at $P \leq 0.05$.

252

253

RESULTS

Impact of Clock Δ19 mutation on mammary development in late pregnancy. To explore the impact of loss of functional circadian timing system on mammary development we compared morphology of glands from mutant *Clock-Δ19* female mice with wild type controls on day 16 of pregnancy. Consistent with previous reports (7), we found that although *Clock-Δ19* females bred to wild-type (WT) males became pregnant and delivered offspring, dams had 0-4 pups present in the early postpartum period. Analysis of number of fetuses in utero on pregnancy day 16 showed no difference in litter size between WT and *Clock-Δ19* mice (7.18± 0.95 and 6.88 ±1.36, respectively), indicating that there was a high rate of neonatal mortality in litters of *Clock-Δ19* dams. Using a milking machine designed for rodents (6) we were able to collect milk samples from *Clock-Δ19* females on post-partum day 3. Although milk quantity was not sufficient for compositional analysis, visual inspection revealed a viscous yellow liquid suggesting that gland differentiation was compromised. Compromised differentiation may be due to either failure to fully differentiate or involution changes in gland due to high loss of suckling neonates by day 3 in *Clock-Δ19* line. Comparing mammary development between late pregnant WT and *Clock-Δ19* dams showed that ratio of lumen to epithelial area in mammary tissue from WT dams was significantly greater in WT than *Clock-Δ19* dams, thus indicating that deficiency in circadian clock impairs mammary development (Fig. 1). Basal plasma prolactin (mean across all time points) only tended to be different (P=0.1) between WT (61.7 ± 17.6 ng/ml) and *Clock-Δ19* (28.2 ± 9.3 ng/ml) mice on pregnancy day 16. There was no difference in basal corticosterone levels (98 ± 38.4 ng/ml and 86 ± 5.1 ng/ml, respectively). It is important to note that the larger standard error of mean basal in plasma hormone levels of WT versus *Clock-Δ19* mice reflects temporal differences in WT animals versus a lack of this difference in *Clock-Δ19*. These studies support that circadian clocks impact mammary development, however we do not know if these effects were due to loss of functional CLOCK in central, peripheral (and) or mammary clock.

Impact of decreased Clock expression on growth of HC11 cells. To determine If CLOCK plays a role in regulation of mammary epithelial cell growth, HC11 cells were

transfected with shRNA specific for CLOCK (*shClock*) or negative control scramble sequence (Table 1). Clonal HC11 cells carrying *shClock* sequence 3 (*shClock-3*) expressed *Clock* at *3*0% of control cultures (Fig. 2A), which resulted in significantly depressed levels of CLOCK protein (Fig. 2B-C). HC11 cells carrying scramble shRNA sequence had no significant change in *Clock* mRNA or protein levels (Fig.2).

Seven day growth curve analysis was used to determine the impact of shRNA sequence on HC11 growth. Growth pattern of cells carrying the negative control scramble sequence was not different from WT culture (Fig. 3), and doubling time between days 2 and 4 of culture was also not different ($P > 0.05$) between WT and scramble transfected HC11 cells (36.1 ± 1.5 and 32.2 ± 0.1 h, respectively). However, transfection with sh*Clock* sequence 1 (*shClock-1*) or *shClock-3* significantly reduced doubling times (23.5 ± 4.3 h or 18.5 ± 1.3 h, respectively) relative to WT cultures. The dramatic decline in cell number in *shClock-3* cultures between days 4 and 7 was most likely due to overcrowding of cells.

To investigate the consequence of reduced CLOCK abundance on progression through the cell cycle and cell cycle regulators, *shClock-3* and HC11 cultures were plated, synchronized in the cell cycle by serum deprivation, released from arrest by addition of serum plus EGF, and collected for FACS and q-PCR analysis. Patterns of cell cycle progression were highly similar among three replicate experiments, with graphs of one trial (Fig. 4) representing the 24 h window (8 h to 32 h) used to analyze mRNA expression of the cell cycle regulators CCND1, WEE1 and TP63. Cells transfected with *shClock-3* appeared to be more sensitive to serum starvation, with 91.1 ± 2.1% of cells arrested in G1 phase of cell cycle at time 0, versus 72.4 ± 13.3% in WT cultures (Fig. 4A). Consistent with a greater rate of growth was evidence for a more rapid succession from S to G2 phase, as there were closer and sharper peaks in these phases across the 24 h period in *shClock-3* cultures compared to WT (Fig. 4B).

Two-way ANOVA of temporal patterns of cell cycle regulator expression showed that time and *shClock-3* significantly impacted CCND1 and WEE1 expression ($P<0.001$). Steady-state levels of CCND1 were elevated in *shClock-3* transfected versus WT cultures across all time points, except during cell cycle arrest (8 h; Fig. 5A). WEE1 expression patterns were shifted and amplitude decreased in cultures

316 transfected with *shClock-3* (Fig. 5B). Further, TP63 mRNA expression was significantly
317 decreased ($P<0.05$) by *shClock-3* across all time points (Fig. 5C). We confirmed that
318 effect of *shClock-3* on cell cycle regulators was due to decreased CLOCK by measuring
319 expression of CCND1 in *shClock-1* and scramble lines. Similar to *shClock-3*, CCND1
320 levels were significantly elevated in *shClock-1* lines, but CCND1 levels were not
321 different between WT and scramble cultures (data not shown).

322 ***Impact of shClock on markers of mammary differentiation in HC11 cells.*** Our
323 previous studies (4, 5, 30) and poorer survival rates of *Clock-Δ19* litters suggest that
324 circadian clocks may function to regulate differentiation and milk synthesis in the
325 mammary gland. To determine if this is regulated at the level of the mammary clock we
326 measured the impact of *shClock* on expression of e-cadherin (CDH1; Fig. 6), fatty acid
327 synthase (FASN; Fig. 6), beta casein (CSN2; Fig. 6), and TP63 (Fig. 7) in
328 undifferentiated (undiff) and differentiated (diff) HC11 cells.

329 CDH1 functions as an adherens junction protein, and its abundance is increased
330 with differentiation of mammary epithelial cells (5). Q-PCR (Fig. 6) and western blot
331 (Fig. 7) analysis showed that CDH1 mRNA and protein levels were significantly
332 decreased in *shClock-3* transfected HC11 versus WT cells. Cells transfected with
333 *shClock-3* also had significantly lower levels of *Fasn* relative to both undifferentiated
334 and differentiated wild-type cultures, however there was no effect of scramble sequence
335 on steady state levels of *Fasn* mRNA (Fig. 6). Levels of the gene that encodes the milk
336 protein CSN2 were significantly increased in differentiated versus undifferentiated states
337 of HC11 cultures both in WT and shClock lines (Fig. 6). However, there was no
338 difference in expression levels among wild-type, scramble or *shClock*-1 or *shClock*-3
339 transfected cultures.

340 ***Impact of shClock on core clock dynamics.***

341 Our previous studies showed that HC11 cells have a robust clock (5). To determine the
342 impact of *shClock* on other components of the core clock, cells were collected from
343 undifferentiated and differentiated cultures. Similar to previous studies we found that
344 PER2 abundance was significantly less and BMAL1 protein abundance was significantly
345 greater in cultures treated with lactogens for 96 h (diff) versus undifferentiated wild-type
346 HC11 cultures (undiff; Fig. 8). However, abundance of PER2 was not different among

undifferentiated and shClock-3 cultures treated with lactogens for 96 h (diff) (Fig. 8). In the *shClock-3* lines, similar to wild type HC11 cultures, BMAL1 abundance was greater in lactogen treated cultures (diff) relative to undifferentiated shClock-3 treatment (Fig. 8).

DISCUSSION

Our analysis of mammary morphology of *Clock-Δ19* mice in late pregnancy showed poorer gland development. Poor pup survival and viscous-yellow mammary secretion of lactating dams also suggested mammary differentiation in *Clock-Δ19* was negatively impacted. Others found that maternal behavior and serum prolactin levels are altered in *Clock-Δ19* dams, however, these alterations are not likely to wholly cause the decreased lactation competency observed in this line (14) (13). Our studies with HC11 cell model system, support that the impaired lactation competency observed in the *Clock-Δ19* mice (7, 14, 15), may be due to lack of a functional clock in mammary epithelial cells.

Decreased CLOCK abundance resulted in increased mammary epithelial cell growth rate, decreased cell line doubling time, and a greater rate of progression through cell cycle. Cells transfected with *shClock* were also more responsive to serum starvation, suggesting that a fully functional clock may buffer against external change. This supposition is supported by studies that have shown tissue-specific rhythmicity can regulate the responsiveness of peripheral clocks to various stimuli, including temporal information from the SCN (24, 32). Changes in growth dynamics were accompanied by differential regulation of the cell cycle regulators WEE1, CCND1 and TP63. A strong correlation between the timing of clock gene expression with the timing of cell-cycle events has been demonstrated in regenerating liver (21) and continuously proliferating tissues (27). The coupling of circadian clock network with cell cycle network occurs through both direct and indirect control of the clock on cell cycle regulators. WEE1 is a direct transcriptional target of BMAL1-CLOCK (21). In our study, temporal pattern of WEE1 expression showed peak levels coincident with timing of transition from G1 to S and G2 to M in both wild-type and *shClock* transfected cells, which is consistent with its cell cycle regulatory role (8). However, decreasing CLOCK levels in HC11 cells resulted in decreased WEE1 amplitude, supporting that known CLOCK-BMAL1 cell cycle regulatory targets were impacted by *shClock* transfection.

Previous studies have shown an association among circadian clock disturbances and changes in CCND1 expression. In *Per2* mutant mice CCND1 temporal expression is altered (11), and osteoblasts isolated from PER1 and PER2

knockout mice exhibited shorter cell cycles and increased growth rates, which was accompanied by higher expression of CCND1 (8). In our study we found reduced CLOCK abundance in HC11 cells was associated with higher expression levels of CCND1, which is consistent with greater growth rates and increased rate of progression through cell cycle measured in *shClock* transfected lines.

Also consistent with greater growth rate and increased rate of progression through cell cycle in *shClock* transfected lines was the dramatically reduced levels of TP63 mRNA and protein levels. TP63 encodes two main isoforms, TAp63 and ΔNp63 both of which are expressed in the mammary gland (10). TAp63 isoforms can bind to DNA through canonical p53-responsive elements and activate the transcription of many p53 target genes. As a result, TAp63 can act as a tumor suppressor in a similar manner to p53 including induction of apoptosis, cell cycle arrest, and senescence (22). ΔNp63 is selectively distributed in the basal cells of stratified and glandular epithelia, and believed to be required to maintain the self-renewing capacity of stem cells in epithelial tissues as well as drive the differentiation program of luminal cells (10, 22). The antibody used for analysis of TP63 protein levels in cells and tissue specifically targeted the transactivation domain of TP63. Therefore, the finding that lower levels of p63 transactivating isomer are associated with increased growth rate and more rapid progression through the cell cycle in *shClock* transfected cells is expected.

We also analyzed the impact of decreased abundance of CLOCK on differentiation of *shClock* transfected HC11 cells, including the expression of e-cadherin (CDH1), fatty acid synthase (FASN), and beta-casein (CSN2). CDH1 functions as an adherens junction protein. Ductal and alveolar epithelial cells express CDH1 in the mammary and its abundance is increased with differentiation of the gland (17). When the function of CDH1 is disrupted in adult mouse mammary gland by means of induction of a mammary specific dominant negative CDH1 molecule, there is massive apoptosis of alveolar cells at parturition with concomitant loss of milk production (3). Temporal analysis of CDH1 steady state mRNA levels in rat kidney showed it exhibits a circadian rhythm of gene expression (34), and thus supports that CDH1 is a direct or indirect target of circadian clock regulation. In our study we found that abundance of CDH1 mRNA and protein levels were dramatically decreased in HC11 cells transfected with

shClock. Thus disruption of clocks may negatively impact formation of adherens junctions among mammary epithelial cells and result in poorer milk production.

Reducing CLOCK levels in HC11 cells significantly decreased expression of FASN in both undifferentiated and differentiated states. FASN encodes the fatty acid synthase enzyme which regulates milk-fat synthesis. Our previous studies found that FASN showed a circadian pattern of expression in wild-type mice during lactation (5). We also found disrupting the circadian timing system by continuously shifting light-dark cycles decreased milk-fat yield in dairy cattle (4). Together these data support that FASN is a direct or indirect target of the BMAL1-CLOCK transcription factor in the mammary gland, and that disruption of circadian clocks has a negative impact on milk fat synthesis, which may explain in part the poorer lactation performance of *Clock-Δ19 mice*.

Reducing CLOCK abundance in HC11 cells did not impact CSN2 expression. CSN2 encodes for the milk protein β-casein, and this finding suggests that the mammary clock does not directly regulate its expression. This is an interesting finding, as our studies of the impact of light-dark phase shifts on milk production in dairy cows showed decreased levels of CSN2 expression in mammary, suggesting that environmental disruption of circadian system may impact its regulation(4). Others reported that milk protein levels show circadian rhythms that are impacted by timing of feed intake (29), and thus daily variation in substrate availability may affect milk protein synthesis. Together these studies illustrate the complexity of the circadian system from the role of local clocks, to feeding time, to systemic influences on timing of circadian rhythms and gene expression.

It is also important to discuss implications of model used in our studies that aimed to understand the role of CLOCK in the regulation of mammary epithelial growth and differentiation. Due to their importance in physiology, adaptation and survival, most of the core circadian clock genes exist as paralogs in mice (*Per1* and *Per2*, *Cry1* and Cry2; and *Clock* and *Npas2*), and *in vivo* studies have shown that both genes of the pair must be knocked out to confer arhythmicity in the animals (18). Further, the inter-related/inter-regulated nature of the transcription-translation feedback-loop that defines the circadian clock mechanism, predisposes that the knock-down or knock-out of one

element affects dynamics of others. Western blot analysis of PER2 and BMAL1 found only modest differences in levels between wild-type HC11 and shCLOCK lines. However, the dynamic changes that occurred in BMAL1 and PER2 proteins following 96 h of lactogen treatment were impacted by shCLOCK-3, with lack of a decrease in PER2 abundance after lactogen treatment. Thus our findings on the impact of decreased CLOCK abundance on HC11 cell growth and differentiation may not be specifically due to alterations in CLOCK, but also changes in mammary circadian clock functional activity. Further, studies in a culture system eliminate systemic influences and feedback mechanisms that may be impacted or maintained in an organismal system, and thus future, more comprehensive studies are needed to understand the role of circadian clocks in regulation of lactation competency.

Conclusion. Our previous studies and work of others demonstrated that the mammary clock shows dynamic changes with changes in physiology from pregnancy to lactation. As the gland transitions from pregnancy to lactation it changes from a primarily proliferative state to a differentiated state. Dynamic changes during the transition from pregnancy to lactation are marked by a relative increase in BMAL1-CLOCK abundance across the circadian cycle. Here we demonstrated that CLOCK plays a role in regulation of mammary epithelial growth and differentiation, and report that when relative abundance of CLOCK is low, proliferation rates of mammary epithelial cells are high and levels of differentiation markers are decreased. Thus supporting the hypothesis that dynamic changes in mammary core clock genes associated with physiological state change regulate developmental changes in the gland and states of proliferation and differentiation, and alterations in clock dynamics may effect lactation competency.

ACKNOWLEDGEMENTS

Grant support:

Binational Agricultural Research Development (BARD) Research Project US-4715-14; Photoperiod effects on milk production in goats: Are they mediated by the molecular clock in the mammary gland?

NIH-R03 1R03HD062692-01A1; Do circadian clocks coordinate physiological changes necessary for milk synthesis?
PDT (Purdue Development team) Award; Determination of mammary circadian clock inputs and outputs.
FPU 12/06079 Scholarship from Ministry of Education, Culture and Sports of the Spanish Government (Madrid, Spain)
The researchers would like to thanks Dr. Marina Antoch of Roswell Park Institute for help and support with animal experiments performed.

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FIGURE CAPTIONS.

Figure 1. Genetic knockout of functional circadian clocks negatively impacts mammary development in *Clock-Δ19* mice. Hematoxylin & eosin (H&E) stained 7 μm sections of mammary tissue collected on pregnancy day 16 from (A) WT (n=6) and (B) *Clock*-Δ19 (n=6) mice show that lack of functional circadian clocks impact mammary differentiation including decreased formation of fat droplets (arrow) and (C) lower ratio of lumen to total epithelial area, measured using image analysis software (Image ProPlus). Student t-test analysis indicated ratio of lumen to epithelial was significantly different between WT and *Clock*-Δ19 mammary glands, $P<0.05$.

Figure 2. Impact of shRNA sequence on CLOCK mRNA and protein levels relative to wild-type (WT) cultures. Q-PCR analysis was used to determine the percent CLOCK mRNA levels in clonal cells lines established after transfection with shRNA sequences 1, 2, and 3 (shClock-1, -2, -3, respectively) or negative control sequence (scramble) relative to wild type (WT) HC11 cultures (A). Representative western blots of CLOCK and beta-actin (BA) proteins; dashed-vertical lines indicate splicing of image to remove intervening lanes within same blot, solid-vertical lines indicate splicing between two different blots (B). Densitometric analysis of western blots and relative ratios of CLOCK: BA proteins across three experiments per clonal line (C). Different letters indicate difference at $P<0.05$ as determined by ANOVA analysis followed by Tukey's post-hoc test.

Figure 3. Growth curve analysis of the impact of shRNA sequence on HC11 cells in culture. On day 0, 100,000 cells were plated in 35 mm dishes and number of cells were counted on days 2, 4, and 7 after plating. Two-way ANOVA analysis of data from three experiments showed that day and line (WT: dashed; shClock-1, dotted; shClock-3, solid black; scramble, solid gray) had a significant effect ($P<0.05$) on growth. Tukey's post-hoc test indicated cell number were different from control at $P<0.05$ (*).

Figure 4. Cell cycle analysis of the impact of shCLOCK on progression of HC11 cells through cell cycle. WT (dashed) and shCLOCK-3 (solid) lines were seeded in 35 mm dishes and allowed to plate for 24 h in growth media. Cultures were serum starved for 24 h, and then growth media (10% serum + EGF) was replaced (time 0). Cultures were collected every 4 h over a 48 h period, and 1.5 million cells were fixed, stained with PI, and stage of cell cycle was determined using FACS. Graphs show difference of percent of WT and shCLOCK-3 lines in G1 phase (A) or S phase (black lines) and G2 phase (gray lines) (B) in a 24 h period, spanning from 8 h to 32 h, post addition of serum +EGF. Data representative of one experiment; experiment was repeated three times.

Figure 5. Impact of shCLOCK on expression of cell cycle regulators. WT and shClock-3 cells were plated in media with 10% serum, serum starved to synchronize cells, and then media with 10% serum + EGF was added (time 0). Cells were collected every 4 h over 48 h. RNA was isolated at from cultures collected during 8 h to 32 h period and Q-PCR analysis was used to measure steady state CCND1 (A), WEE1 (B) and TP63 mRNA levels. Two-way ANOVA of data from three replicate experiments showed that time and line (WT versus shClock-3) had significant ($P<0.001$) impacts on CCND1 and WEE1 expression; and line had a significant ($P<0.001$) impact of TP63 expression.

Figure 6. Impact of shCLOCK on markers of differentiation in HC11 cells. WT, scramble and shClock-3 cells were plated and grown to confluence in 10% + EGF, media was change to 10% serum alone and undifferentiated (undiff) cultures were collected after 48 h. Differentiated (diff) cultures were incubated for an additional 96 h in media supplemented with dexamethasone, Ins, and prolactin + 10% serum. RNA was isolated and Q-PCR analysis was used to measure steady state CDH1 (A), FASN (B), and CSN2 (C) mRNA levels. Two-way ANOVA of three replicate experiments showed that state of differentiation had a significant impact on CDH1, FASN, and CSN2 expression and line (WT HC11, scramble, shClock-3) had significant ($P<0.05$) effect on CDH1 and FASN expression. Tukey's post-hoc analysis demonstrated that expression

level was different from HC11-undiff (indicated with an asterisk, *), or from HC11-diff (indicated with “a”) at *P*<0.05.

Figure 7. Impact of shClock on CDH1 and TP63 protein levels in undifferentiated (undiff) and differentiated (diff) HC11 cells. WT (white bar), and shClock-3 (black bar) cells were plated and grown to confluence in 10% + EGF, media was change to 10% serum alone and undifferentiated (undiff) cultures were collected after 48 h. Differentiated (diff) cultures were incubated for an additional 96 h in media supplemented with dexamethasone, Ins, and prolactin + 10% serum. Protein lysates were prepared and used for western blot analysis of CDH1 (A) and TP63 (B) levels. Two-way ANOVA analysis of data from three experiments indicated that state of differentiation and line had significant effects ($P \leq 0.05$) on both CDH1 and TP63 levels. Tukey’s post-hoc analysis demonstrated that expression level was different from HC11-undiff (indicated with an asterisk, *), or from HC11-diff (indicated with “a”) at *P*<0.05.

Figure 8. Impact of shClock on PER2 and BMAL1 protein abundance in undifferentiated and differentiated HC11 cells. WT and shClock-3 cells were plated and grown to confluence in 10% + EGF, media was change to 10% serum alone and undifferentiated (undiff) cultures were collected after 48 h. Differentiated (diff) cultures were incubated for an additional 96 h in media supplemented with dexamethasone, Ins, and prolactin + 10% serum. Protein lysates were prepared and used for western blot analysis of beta actin (BA) and PER2 (A) and BMAL1 (B) levels; dashed-vertical lines indicate splicing to remove intervening lanes within same blot, solid-vertical lines indicate splicing between two different blots run simultaneously. Densitometric analysis was used to determine ratio of PER2 and BMAL1 to BA across three replicate experiments. Different letters indicate difference at P<0.05 as determined by ANOVA analysis followed by Tukey’s post-hoc test.

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Table 1: Nucleotide sequence of shRNA transfected into HC11 cells	
Sequence ID	Insert Sequence
shClock-1	AAACCCACATTCCTTAGTAAT
shClock-2	GCAACTTGTGACCAAATTAGT
shClock-3	CGATGTCTCAAGCTGCAAATT
shClock-4	ATCAAACCCTGGATTGAATTT
Negative control (scramble)	ggaatctcattcgatgcatac

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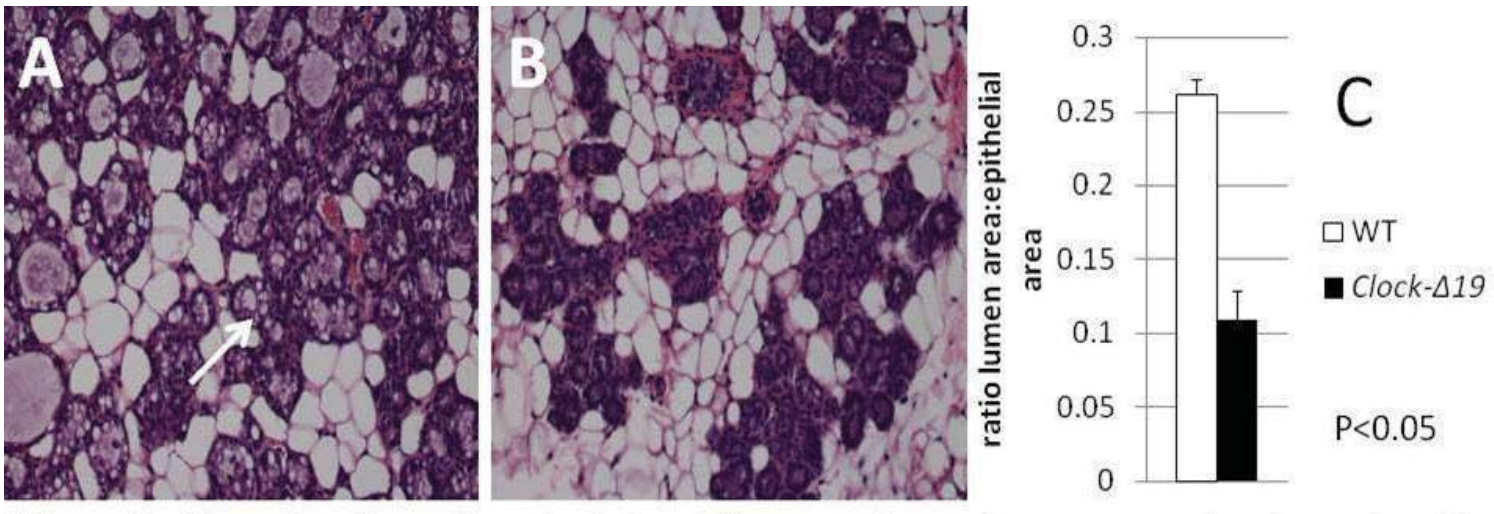
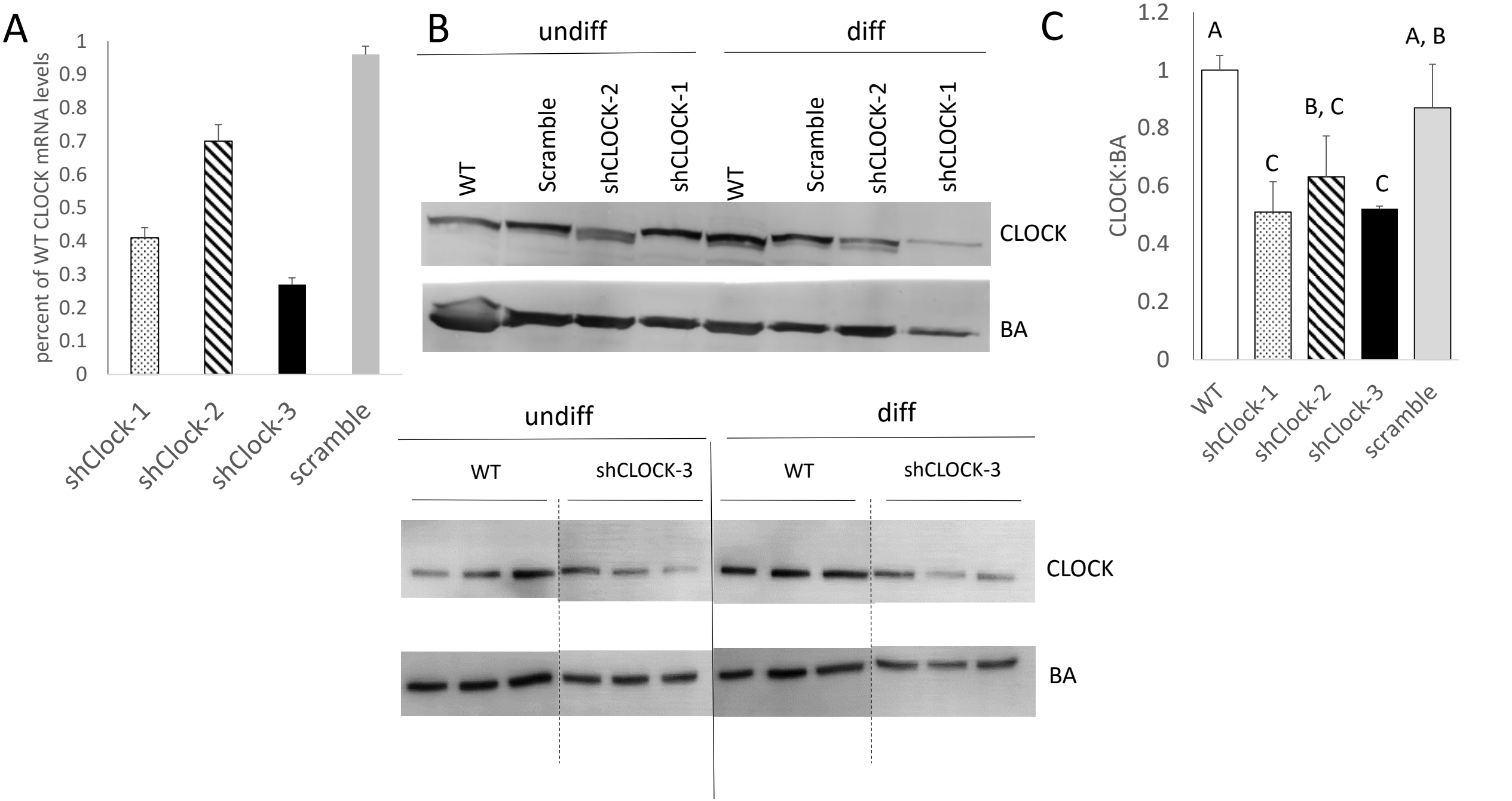
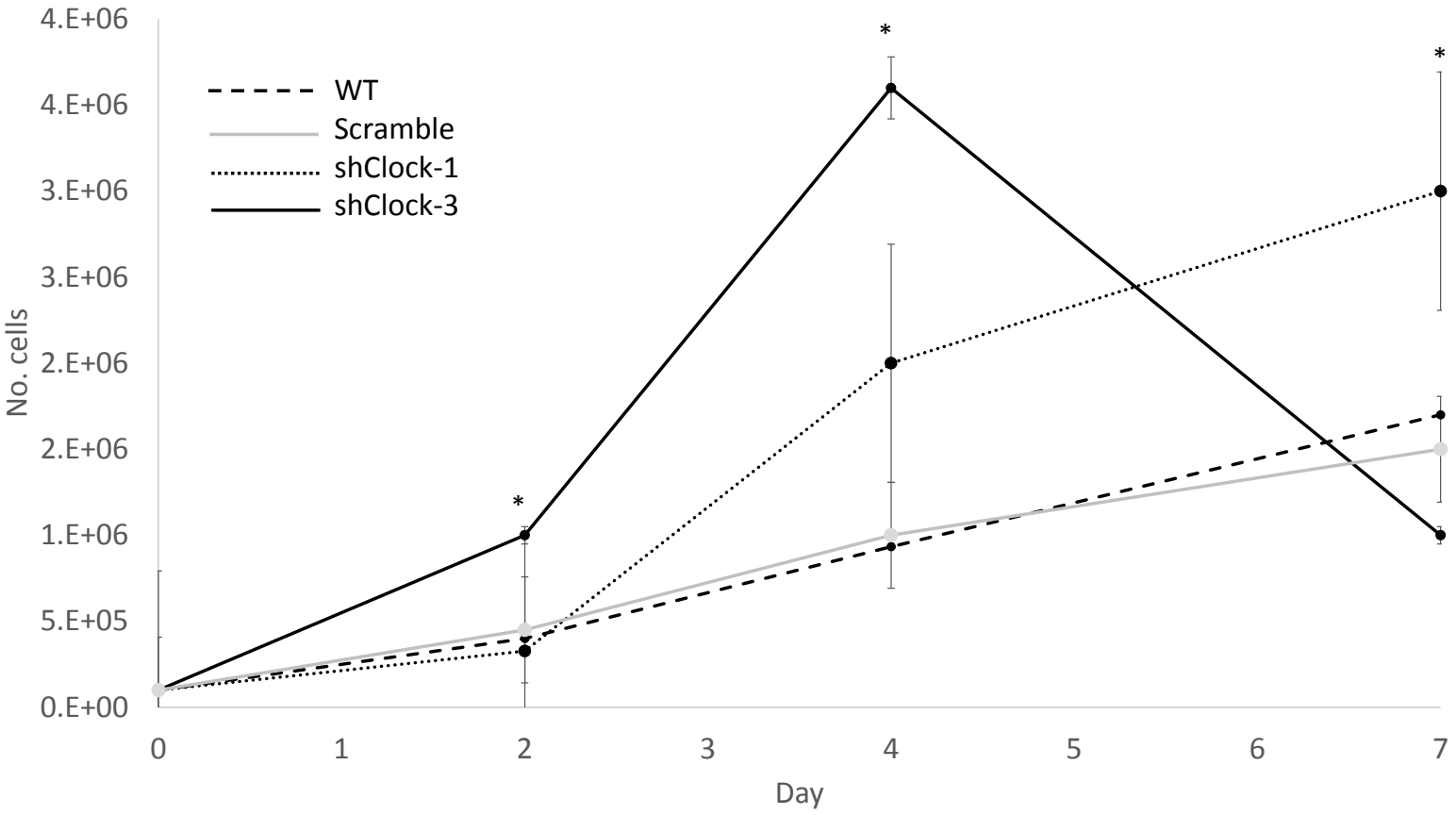
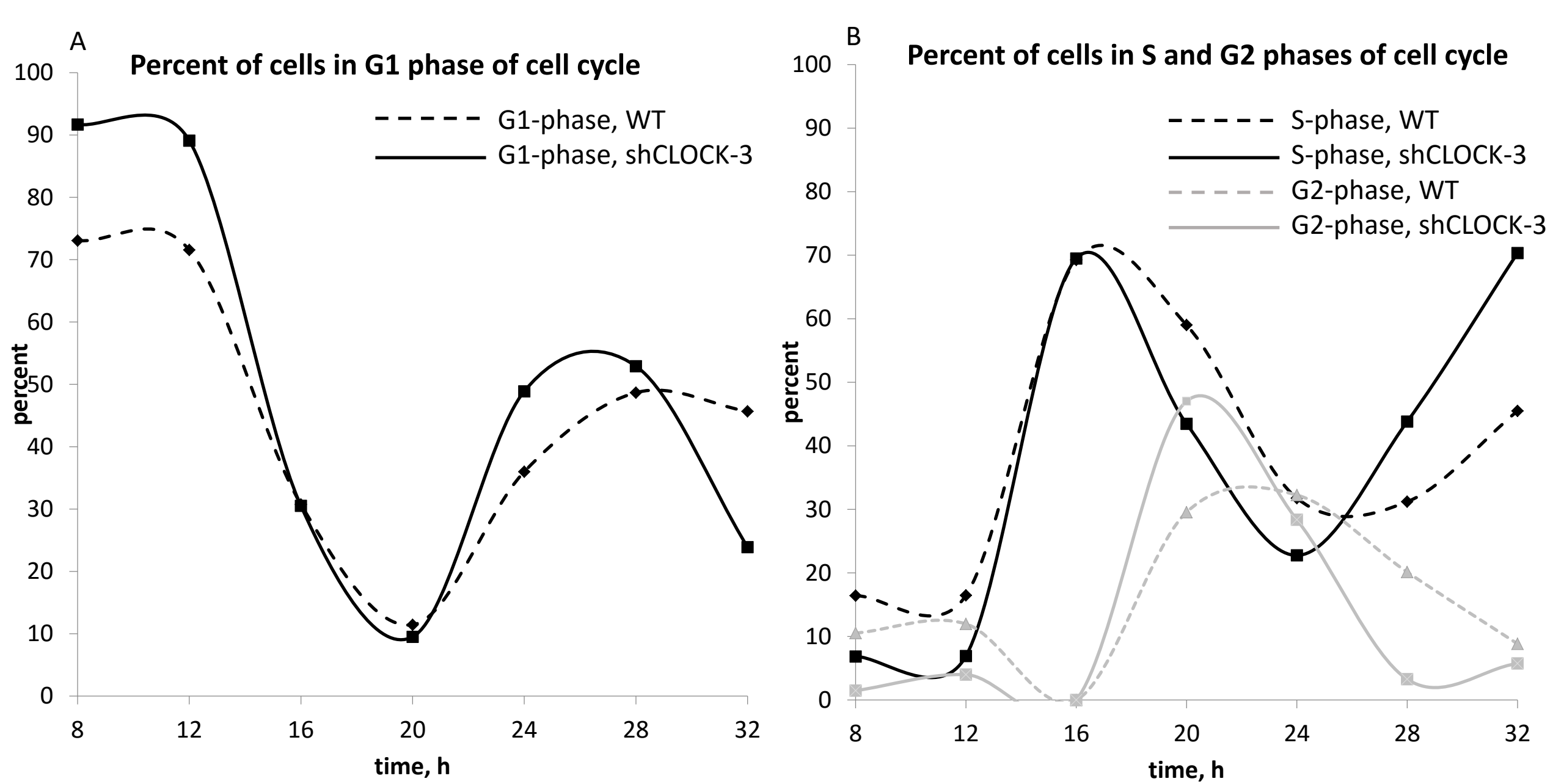
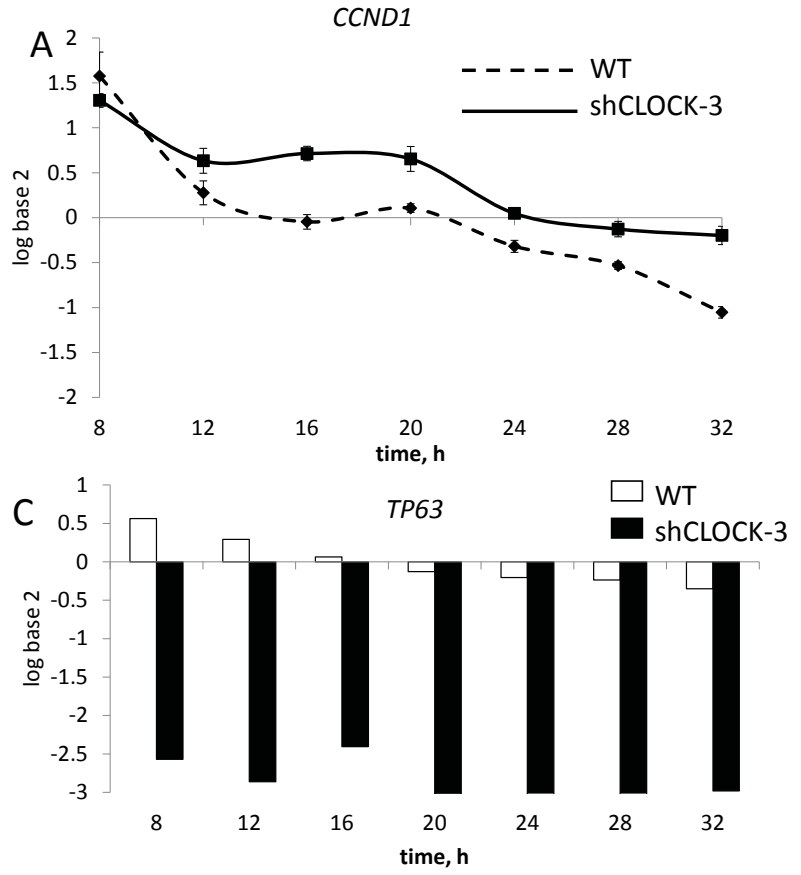
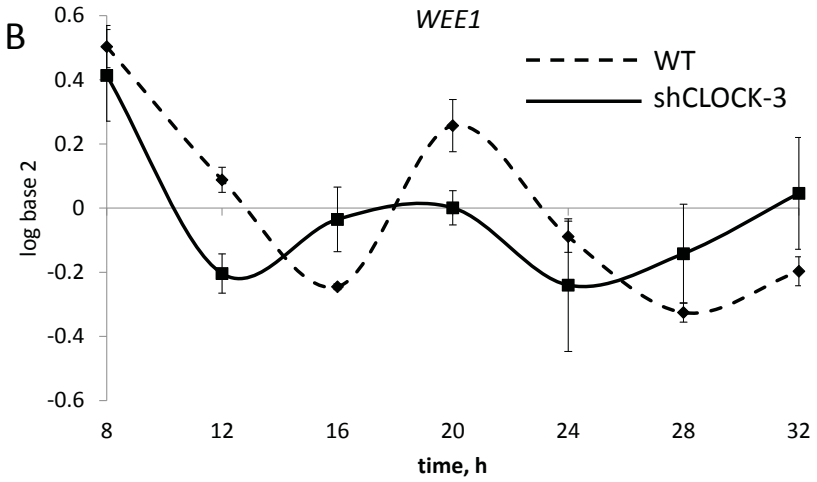

A
B
ratio lumen area:epithelial area
0.3
0.25
0.2
0.15
0.1
0.05
0
C
WT
Clock-Δ19
P<0.05


A
percent of WT CLOCK mRNA levels
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
shClock-1
shClock-2
shClock-3
scramble
B
undiff
diff
WT
Scramble
shCLOCK-2
shCLOCK-1
WT
Scramble
shCLOCK-2
shCLOCK-1
CLOCK
BA
undiff
diff
WT
shCLOCK-3
WT
shCLOCK-3
CLOCK
BA
C
CLOCK:BA
1.2
1
0.8
0.6
0.4
0.2
0
A
C
B, C
C
A, B
WT
shClock-1
shClock-2
shClock-3
scramble

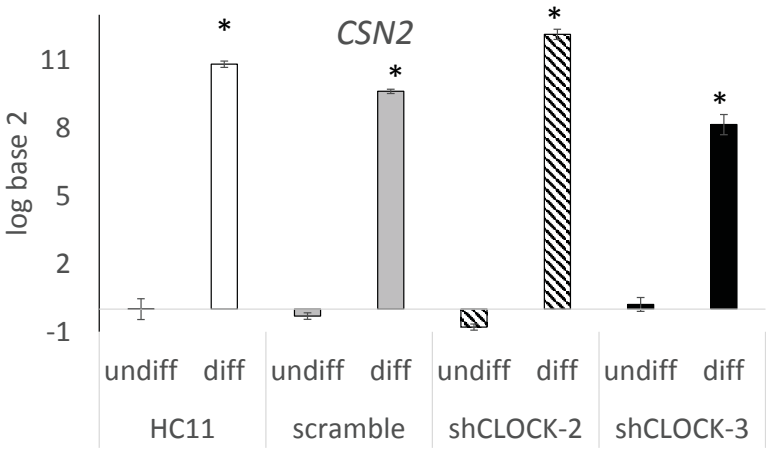

4.E+06
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
No. cells
0
1
2
3
4
5
6
7
Day
WT
Scramble
shClock-1
shClock-3
*
*
*

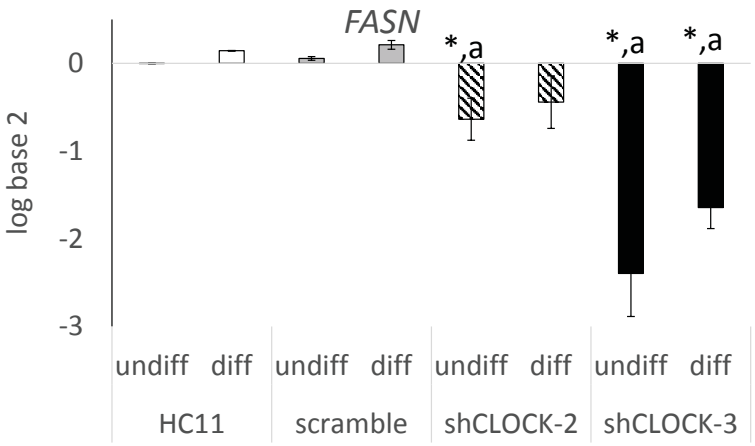

A
Percent of cells in G1 phase of cell cycle
G1-phase, WT
G1-phase, shCLOCK-3
percent
time, h
B
Percent of cells in S and G2 phases of cell cycle
S-phase, WT
S-phase, shCLOCK-3
G2-phase, WT
G2-phase, shCLOCK-3
percent
time, h

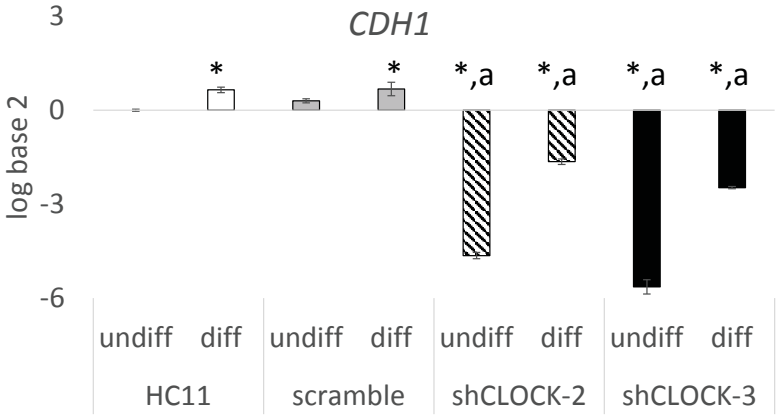

A
CCND1
WT
shCLOCK-3
log base 2
time, h
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
8
12
16
20
24
28
32


B
WEE1
WT
shCLOCK-3
log base 2
time, h
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
8
12
16
20
24
28
32

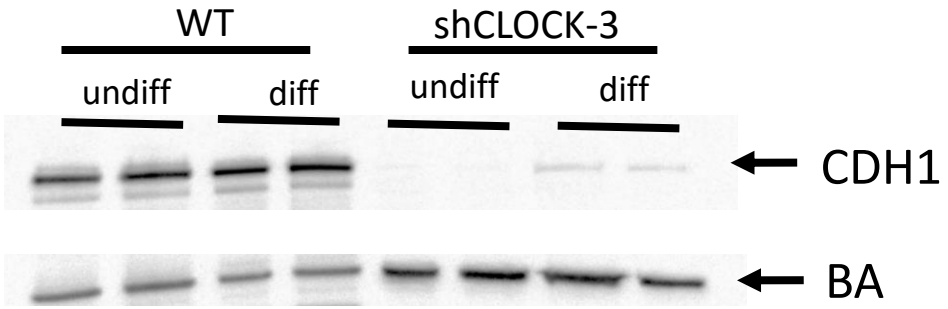
C
TP63
WT
shCLOCK-3
log base 2
time, h
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
8
12
16
20
24
28
32

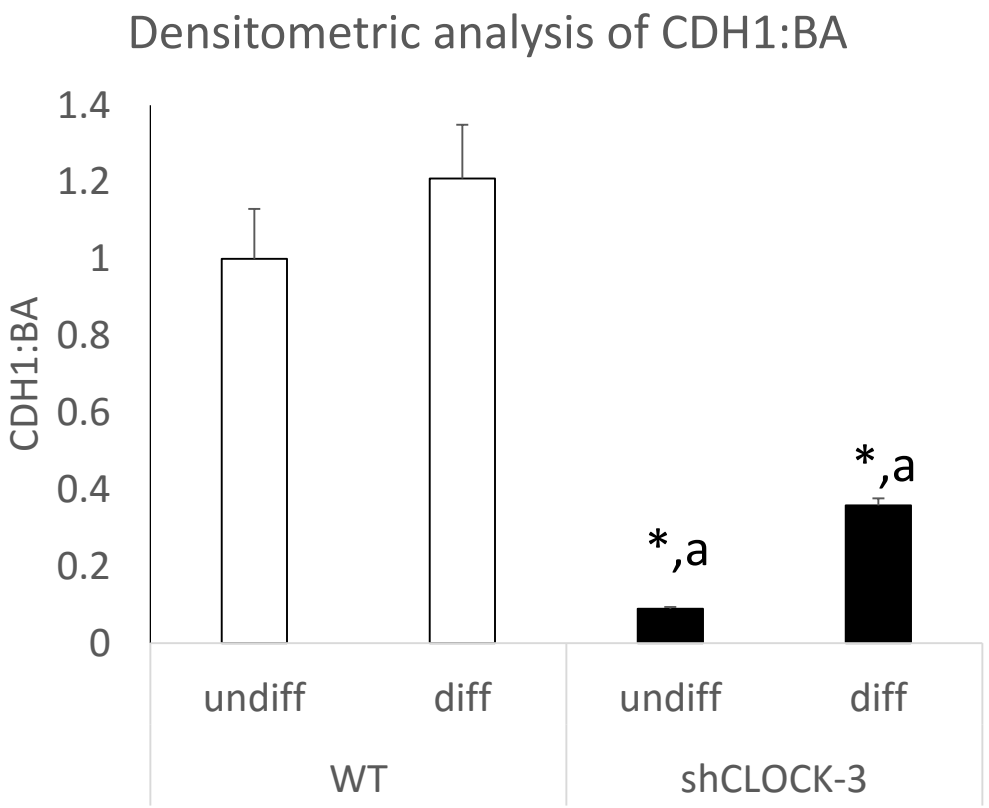

CSN2
log base 2
11
8
5
2
-1
*
*
*
*
undiff diff undiff diff undiff diff undiff diff
HC11
scramble
shCLOCK-2
shCLOCK-3


FASN
log base 2
0
-1
-2
-3
*,a
*,a
*,a
undiff diff undiff diff undiff diff undiff diff
HC11
scramble
shCLOCK-2
shCLOCK-3

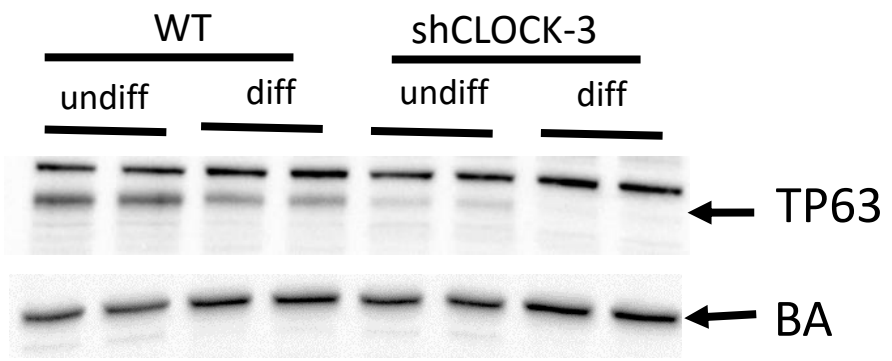

CDH1
log base 2
3
0
-3
-6
*
*
*,a
*,a
*,a
*,a
undiff diff undiff diff undiff diff undiff diff
HC11
scramble
shCLOCK-2
shCLOCK-3

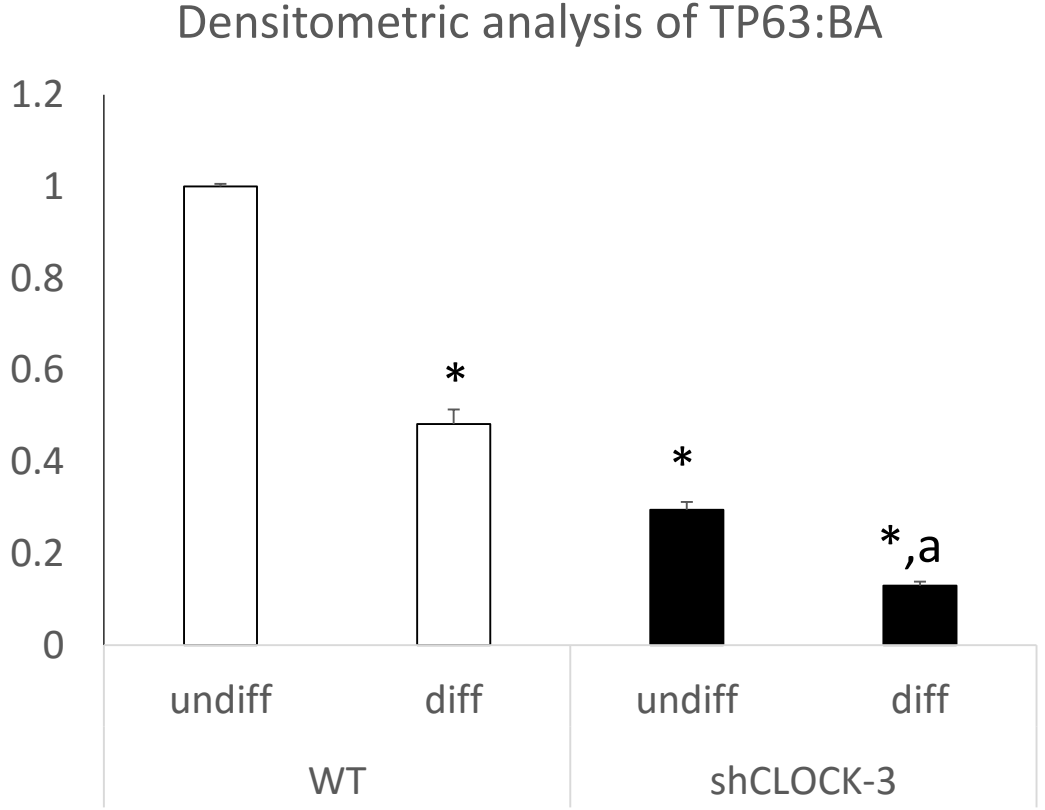
A

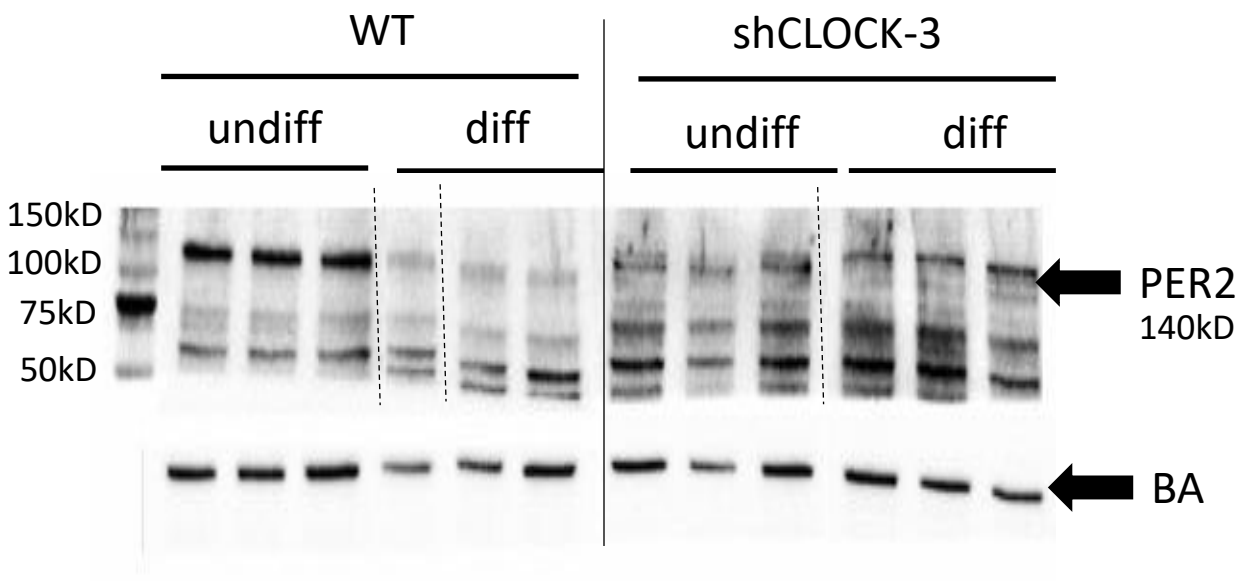

WT
shCLOCK-3
undiff
diff
undiff
diff
CDH1
BA

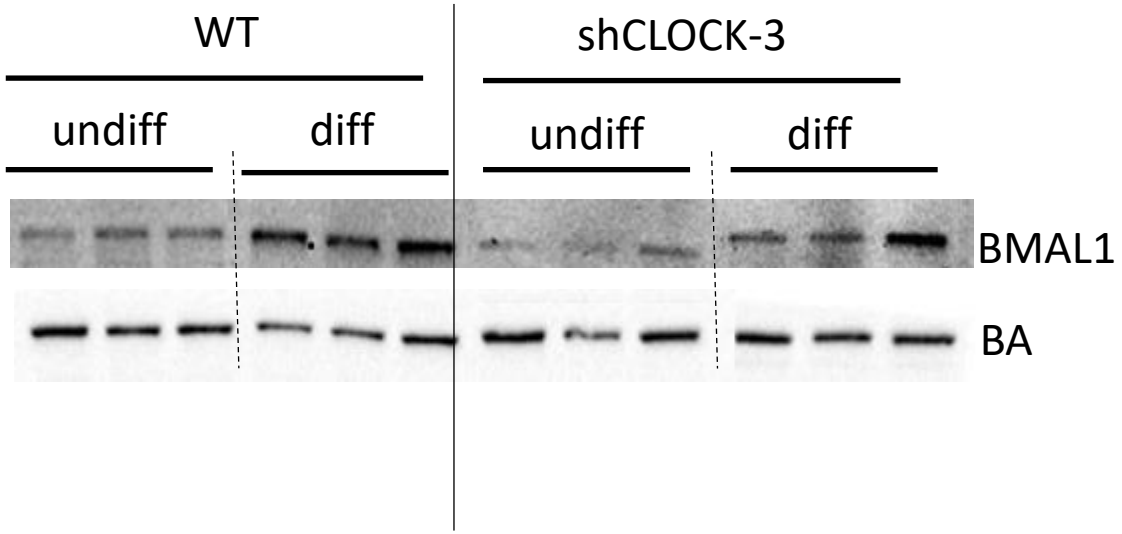

Densitometric analysis of CDH1:BA
CDH1:BA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*,a
*,a
undiff
diff
undiff
diff
WT
shCLOCK-3

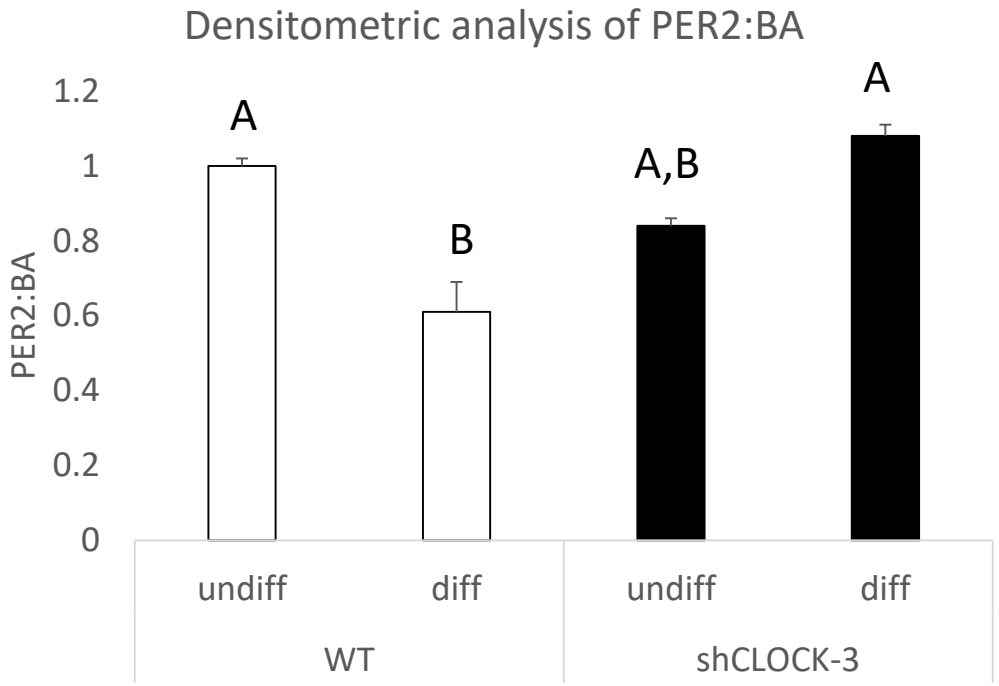
B

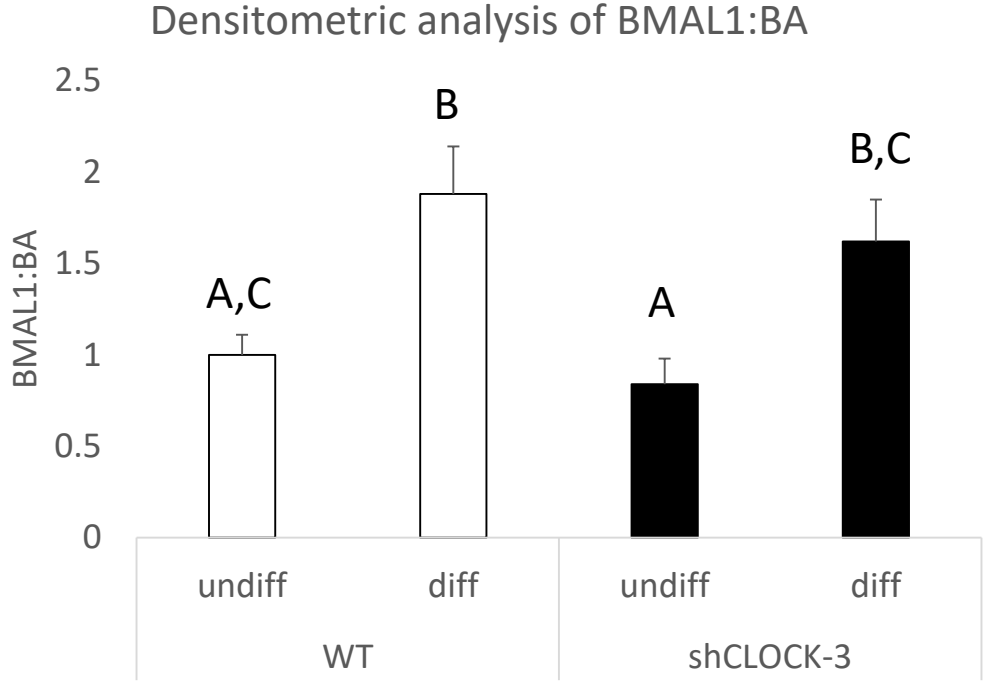

WT
shCLOCK-3
undiff
diff
undiff
diff
TP63
BA


Densitometric analysis of TP63:BA
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*,a
undiff
diff
undiff
diff
WT
shCLOCK-3


WT
shCLOCK-3
undiff
diff
undiff
diff
150kD
100kD
75kD
50kD
PER2
140kD
BA


WT
shCLOCK-3
undiff
diff
undiff
diff
BMAL1
BA


Densitometric analysis of PER2:BA
PER2:BA
1.2
1
0.8
0.6
0.4
0.2
0
A
B
A,B
A
undiff
diff
undiff
diff
WT
shCLOCK-3


Densitometric analysis of BMAL1:BA
BMAL1:BA
2.5
2
1.5
1
0.5
0
A,C
B
A
B,C
undiff
diff
undiff
diff
WT
shCLOCK-3