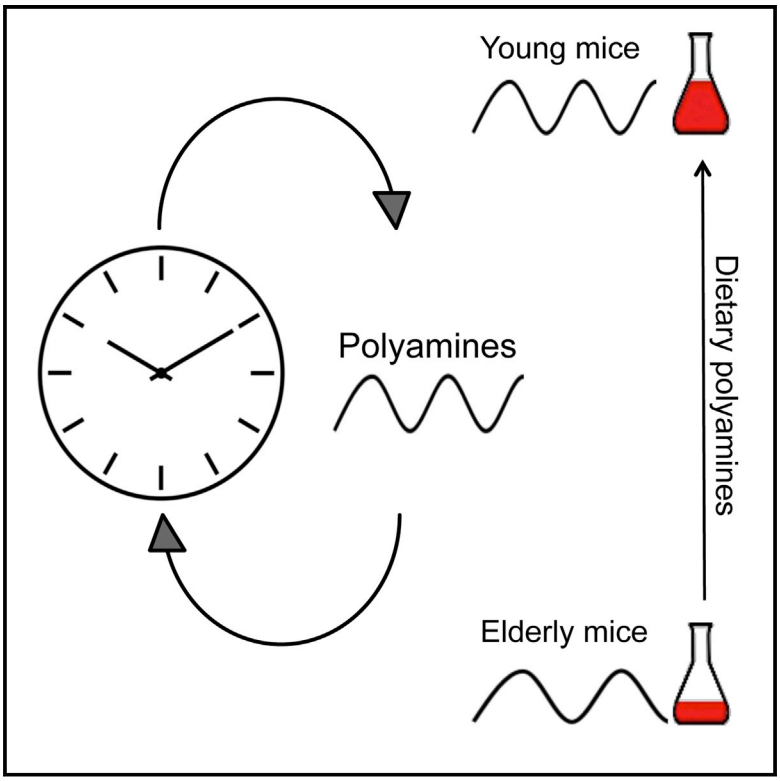
Article

Cell Metabolism

Circadian Clock Control by Polyamine Levels through a Mechanism that Declines with Age

Graphical Abstract



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In Brief

Polyamines are regulated essential molecules participating in key cellular processes, e.g., transcription. Zwighaft et al. identify a reciprocal metabolic feedback loop coupling circadian clock and polyamine biosynthesis. Aging-associated decline in polyamine levels lead to a correspondingly longer circadian period, which can be reversed by dietary polyamine supplementation.

Highlights

- Diurnal regulation of polyamine metabolism by circadian clocks and feeding
- Polyamine levels regulate the circadian period in cultured cells and mice
- Polyamines modulate the interaction between the core clock proteins PER2 and CRY1
- Lengthening of the circadian period with age can be reversed by polyamines

Zwighaft et al., 2015, Cell Metabolism *22*, 1–12
November 3, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cmet.2015.09.011

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Please cite this article in press as: Zwighaft et al., Circadian Clock Control by Polyamine Levels through a Mechanism that Declines with Age, Cell Metabolism (2015), http://dx.doi.org/10.1016/j.cmet.2015.09.011

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Article

Circadian Clock Control by Polyamine Levels through a Mechanism that Declines with Age

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http://dx.doi.org/10.1016/j.cmet.2015.09.011

SUMMARY

Polyamines are essential polycations present in all living cells. Polyamine levels are maintained from the diet and de novo synthesis, and their decline with age is associated with various pathologies. Here we show that polyamine levels oscillate in a daily manner. Both clock- and feeding-dependent mechanisms regulate the daily accumulation of key enzymes in polyamine biosynthesis through rhythmic binding of BMAL1:CLOCK to conserved DNA elements. In turn, polyamines control the circadian period in cultured cells and animals by regulating the interaction between the core clock repressors PER2 and CRY1. Importantly, we found that the decline in polyamine levels with age in mice is associated with a longer circadian period that can be reversed upon polyamine supplementation in the diet. Our findings suggest a crosstalk between circadian clocks and polyamine biosynthesis and open new possibilities for nutritional interventions against the decay in clock's function with age.

INTRODUCTION

Polyamines (i.e., putrescine, spermidine, and spermine) are essential ubiquitous polycations present in all living organisms. They participate in the regulation of various key cellular processes such as chromatin structure, gene transcription and translation, cell growth, and proliferation. Polyamines are believed to exert their effects through modulating protein-protein and protein-DNA interactions. Cellular polyamine levels are maintained within a narrow physiological range and are tightly regulated through uptake/secretion, de novo synthesis/catabolism, and inter-conversion. Altered polyamine metabolism is associated with various pathologies such as neurological abnormalities, malignancies, and aging (Moinard et al., 2005; Pegg, 2009).

Circadian clocks orchestrate the daily oscillations in physiology and behavior. The mammalian circadian timing system consists of a central pacemaker in the brain that is entrained by light-dark cycles and synchronizes subsidiary oscillators in virtually all cells of the body in part by driving cyclic feeding behavior. The core clock circuitry relies on interlocked transcription-translation feedback loops. The transcription factors BMAL1 and CLOCK bind as heterodimers to E-box motifs present in the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes and drive their transcription. Subsequently PER:CRY protein complexes accumulate and auto-repress BMAL1:CLOCK-mediated transcription. BMAL1 also activates *Rev-erb* transcription, which in turn suppresses *Bmal1* expression. The coordinated activity of these feedback loops drives cyclic gene expression with an ~24 hr period, both in cultured cells and living animals (Feng and Lazar, 2012; Mohawk et al., 2012).

Compelling evidence points toward an interplay between circadian rhythms and cellular metabolism (Asher and Sassone-Corsi, 2015; Asher and Schibler, 2011). Circadian clocks play a principal role in orchestrating the daily expression of regulators and enzymes involved in nutrient processing and energy homeostasis. Concomitantly, clocks are tightly coupled to cellular metabolism and respond to feeding cycles. However, the molecular mechanisms through which metabolism affects the clock's function are largely unknown. Since polyamine levels are tightly regulated and polyamines facilitate protein-protein/DNA interactions, which are fundamental for the clock's function, we set out to examine: (1) whether circadian clocks regulate cellular polyamine levels, and (2) whether polyamines play a role in the function of the core clock, a conjecture that was so far never tested.

In this study, we uncover a crosstalk between circadian clocks and polyamines. We show that both clock- and feeding-dependent mechanisms regulate the daily oscillations of key enzymes in polyamine biosynthesis and consequently polyamine levels. In turn, we found that polyamines participate in circadian period control in cultured cells and animals. At the molecular level, polyamines modulate the interaction between the repressor members of the core clock circuitry, PER2 and CRY1. Finally, we demonstrate that the decline in polyamine levels with age in mice is associated with a longer circadian period that can be reversed upon dietary polyamine supplementation.

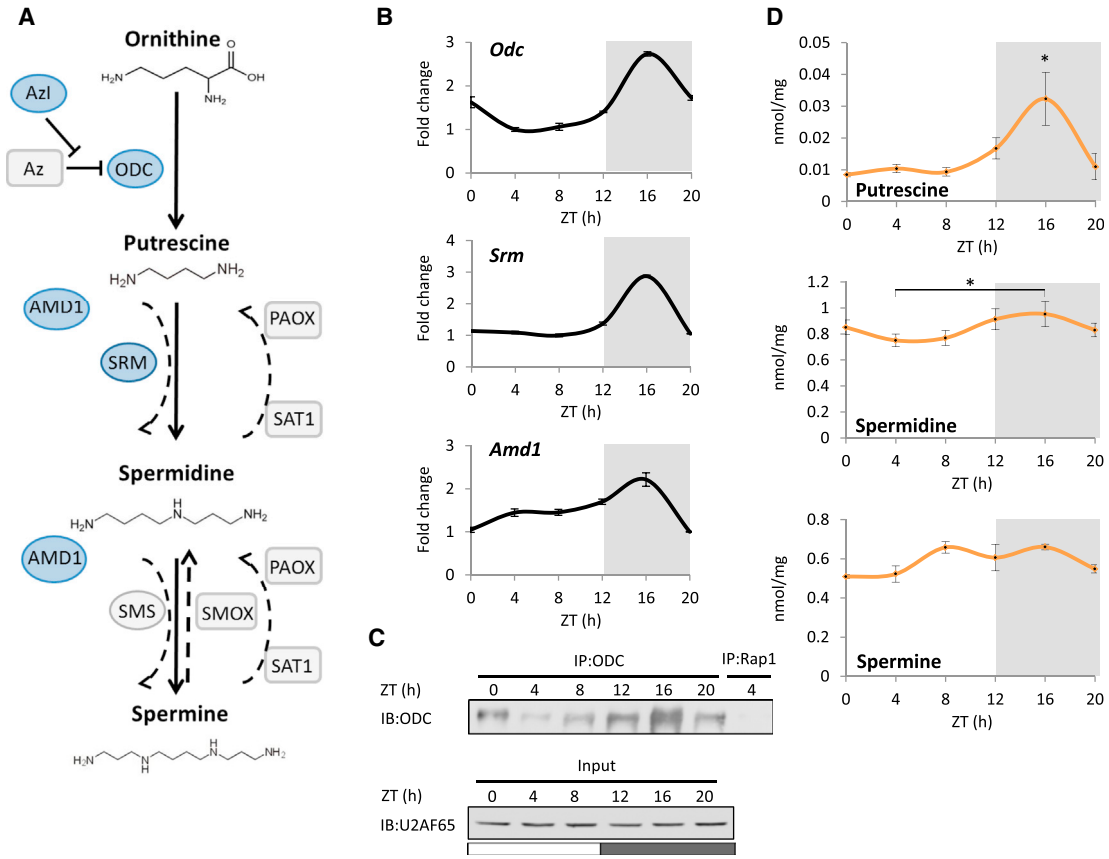

Figure 1. Key Enzymes in Polyamine Metabolism and Polyamine Levels Oscillate in a Daily Manner

(A) A schematic depiction of the polyamine biosynthesis pathway. Ornithine decarboxylase, ODC; spermidine synthase, SRM; spermine synthase, SMS; spermidine/spermine-N1-acetyltransferase, SAT1; spermine oxidase, SMOX; polyamine oxidase, PAOX; adenosylmethionine decarboxylase 1, AMD1; antizyme, Az; and antizyme inhibitor, AzI. Enzymes implicated in polyamine synthesis are in circles and in polyamine catabolism in squares. Enzymes that are expressed in a daily manner are colored in blue (see panel B and Figure S1A).

(B) Wild-type mice were sacrificed at 4 hr intervals throughout the day. Total RNA was prepared from liver and transcript levels of *Odc*, *Srm*, and *Amd1* were determined by quantitative real-time PCR and presented as fold change relative to the lowest value. Data are presented as mean ± SD of 4 mice per time point.

(C) Total liver protein extracts were prepared (Input) and ODC was immunoprecipitated (IP) and analyzed by immunoblot (IB). Rap1 antibody was used as a negative control.

(D) The levels of putrescine, spermidine, and spermine in mouse liver were quantified by HPLC and presented in nmol/mg tissue with mean ± SEM of 4 mice per time point, *$p < 0.05$. Gray shading represents the dark phase; zeitgeber time, ZT. See also Figure S1.

RESULTS

Key Enzymes in Polyamine Metabolism and Polyamine Levels Oscillate in a Daily Manner

Mammals obtain polyamines by de novo synthesis and through uptake from the diet. The de novo polyamine biosynthesis pathway consists of several successive steps (Figure 1A) (Pegg, 2009). The first and rate-limiting step is the decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC). Subsequently, putrescine is converted to spermidine, and the latter is metabolized to spermine. These enzymatic reactions are carried out by the aminopropyltransferases, spermidine synthase (SRM) and spermine synthase (SMS), respectively. The aminopropyl group derives from the decarboxylation of s-adenosylmethionine by adenosylmethionine decarboxylase 1 (AMD1). In addition, cellular polyamine homeostasis is maintained through polyamine catabolism and inter-conversion (Casero and Pegg, 2009). These steps are mediated through the activity of several enzymes including spermidine/spermine-N1-acetyltransferase (SAT1), spermine oxidase (SMOX), and polyamine oxidase (PAOX). Since ODC is the rate-limiting enzyme in polyamine biosynthesis, its levels are tightly regulated. ODC is a short-lived protein that is targeted for degradation by antizyme (Az) through the 26S proteasome in a ubiquitin-independent manner (Kahana et al., 2005) or degraded by the 20S proteasome in an NQO1-regulated manner (Asher et al., 2005). Antizyme inhibitor (AzI) binds Az, preventing it from directing ODC for degradation (Kahana et al., 2005).

First, we asked whether polyamine metabolism exhibit diurnal rhythmicity. Mice were sacrificed at 4 hr intervals throughout the day and liver transcript levels of polyamine metabolic enzymes and core clock genes were quantified (Figures 1B, S1A, and S1B). The majority of enzymes participating in polyamine anabolism were expressed in a daily manner. *Odc*, *Srm*, and *Amd1*

all reached their peak levels ~16 zeitgeber time (~ZT16) (Figure 1B). Notably, their daily expression profile mostly resembled that of *Per2* (Figure S1B). The mRNA levels of *Azl*, which supports polyamine synthesis by stabilizing ODC, also exhibited shallow daily oscillations with peak levels ~ZT12 and trough levels ~ZT0 (Figure S1A). By contrast, the transcript levels of all tested enzymes participating in polyamine catabolism (i.e., *Sat1*, *Smox*, *Paox*), including *Az*, which targets ODC for degradation, were relatively constant throughout the day (Figure S1A). Our analysis evinced that enzymes participating in polyamine anabolism are expressed in a diurnal manner, whereas enzymes involved in polyamine catabolism are mostly constant throughout the day.

To substantiate these findings, we examined the protein levels of ODC, the rate-limiting enzyme in polyamine synthesis, in livers from mice sacrificed throughout the day. In whole liver extracts, ODC was undetectable (data not shown), conceivably, due to its low abundance. Hence, we immunoprecipitated ODC from liver extracts and detected it by immunoblotting. ODC accumulated in a daily manner similar to its transcript levels (zenith and nadir levels ~ZT16 and ~ZT4, respectively) (Figure 1C). Next, we quantified the levels of the different polyamine species in mouse liver (Figure 1D). Putrescine, the product of ODC enzymatic activity cycled and reached its peak levels ~ZT16, in line with *Odc* mRNA and protein accumulation. Spermidine levels oscillated with a similar phase as putrescine but with shallow amplitude, whereas spermine levels were relatively constant throughout the day. Notably, analysis of serum putrescine and spermidine levels showed that putrescine levels are relatively constant throughout the day, while spermidine levels cycle with peak levels ~ZT16 (Figure S1C). Taken together, our data evinced that key enzymes in polyamine biosynthesis (i.e., *Odc*, *Srm*, and *Amd1*) and the polyamines putrescine and spermidine cycle throughout the day with peak levels during the night.

Clock- and Feeding-Dependent Mechanisms Regulate the Daily Expression of Key Enzymes in Polyamine Biosynthesis and Polyamine Accumulation

Next, we examined whether the daily oscillations of key enzymes in polyamine biosynthesis and polyamine accumulation are dependent on a functional clock. We compared the daily expression profiles of polyamine metabolic enzymes in wild-type and *Per1/2* null mice (Figures 2A and S2A). *Per1/2* null mice exhibit arrhythmic behavior in constant darkness and their circadian clock genes' expression is largely diminished (Figure S2B) (Zheng et al., 2001). Notably, in *Per1/2* null mice, *Odc*, *Srm*, and *Amd1* displayed relatively shallow daily oscillations compared to wild-type mice (Figure 2A). Furthermore, putrescine, spermidine, and spermine levels were fairly constant throughout the day (Figure 2B). These results suggested that PER1/2 and thus circadian clocks play a role in their rhythmic accumulation.

Clock mutant mice (e.g., *Per1/2* null) exhibit shallow feeding rhythms and consume relatively equal amount of food throughout the day (Adamovich et al., 2014). Hence, to examine the responsiveness of *Odc*, *Srm*, and *Amd1* expression to feeding, we analyzed the expression profiles of these enzymes and clock genes in wild-type mice fed either exclusively during the night, or solely during the day for 3 weeks. As expected, the expression pattern of clock genes in 3 weeks day-fed animals was completely inverted (Figures 2C and S2C) (Damiola et al., 2000). *Per2* mRNA reached its maximal levels ~ZT4 in day-fed animals compared to ~ZT16 in mice fed either exclusively during the night or ad libitum (Figure 2). The expression profiles of *Odc*, *Srm*, and *Amd1* resembled that of *Per2* (Figure 2C). Thus, similarly to clock genes, principal enzymes in polyamine biosynthesis responded to changes in feeding time. However, since under these conditions both the molecular clock and feeding are in accordance and inverted, it is not possible to conclude whether rhythmic expression is driven by the circadian clock or rather responds directly to feeding. In an attempt to discriminate between these two possibilities, we examined the transcript profiles of *Odc*, *Srm*, *Amd1*, and clock genes during the first day of daytime feeding. During this time window *Bmal1*, *Rev erbα*, and *Dbp* exhibited shallower oscillations that were slightly shifted compared to night-fed animals (Figure S2C). By contrast, *Per2* mRNA exhibited two prominent peaks, one ~ZT16, similar to night-fed animals and an additional peak emerged ~ZT8 (Figure 2C). Conceivably the former is clock dependent, while the latter is driven by feeding. This is in line with previous reports classifying *Per2* as a core clock component that is also systemically regulated, most likely by feeding (Kornmann et al., 2007). Remarkably, *Odc*, *Srm*, and *Amd1* displayed a similar expression pattern as *Per2* under all three tested conditions (Figure 2C). We concluded that both clock- and feeding-dependent mechanisms drive the daily expression of key enzymes in polyamine biosynthesis and polyamine accumulation.

BMAL1:CLOCK Bind to E-Box Motifs within the *Odc* Gene in a Circadian Manner

The resemblance in the expression profiles of *Per2* and *Odc* in mouse liver under different feeding regimens (Figure 2) suggested that they might share similar mechanisms of gene expression regulation. BMAL1:CLOCK heterodimers bind to E-box motifs (CACGTG) present in the *Per* gene and drive its transcription (Mohawk et al., 2012). We identified three canonical E-box motifs in the *Odc* gene, two within its first intron (E-Box In1) and one in the 3′ UTR (E-Box 3′ UTR), (Figure 3A). The two E-box motifs within the first intron are conserved in mammals (Figure 3B) and were previously reported to participate in *Odc* expression by c-Myc (Bello-Fernandez et al., 1993). To determine whether BMAL1 binds these E-boxes, we performed chromatin immunoprecipitation (ChIP) experiments with BMAL1-specific antibodies throughout the day. In agreement with previous studies, BMAL1 exhibited circadian binding to intron 2 but not to exon 4 of the *Dbp* gene, with maximal occupancy ~ZT8 and minimal ~ZT20 (Figure S3A), (Ripperger and Schibler, 2006). Importantly, our ChIP experiments revealed that BMAL1 binds the E-box elements in intron 1 but not in the 3′ UTR region of the *Odc* gene. The binding of BMAL1 to intron 1 of *Odc* oscillated in a daily manner with peak and trough levels ~ZT8 and ~ZT20, respectively (Figure 3C). Similar results were obtained with CLOCK-specific antibodies (Figures 3D and S3B). These results suggested that BMAL1:CLOCK heterodimers bind to E-box motifs within the first intron of the *Odc* gene in a daily manner and hence are likely to drive the rhythmic transcription of *Odc*.

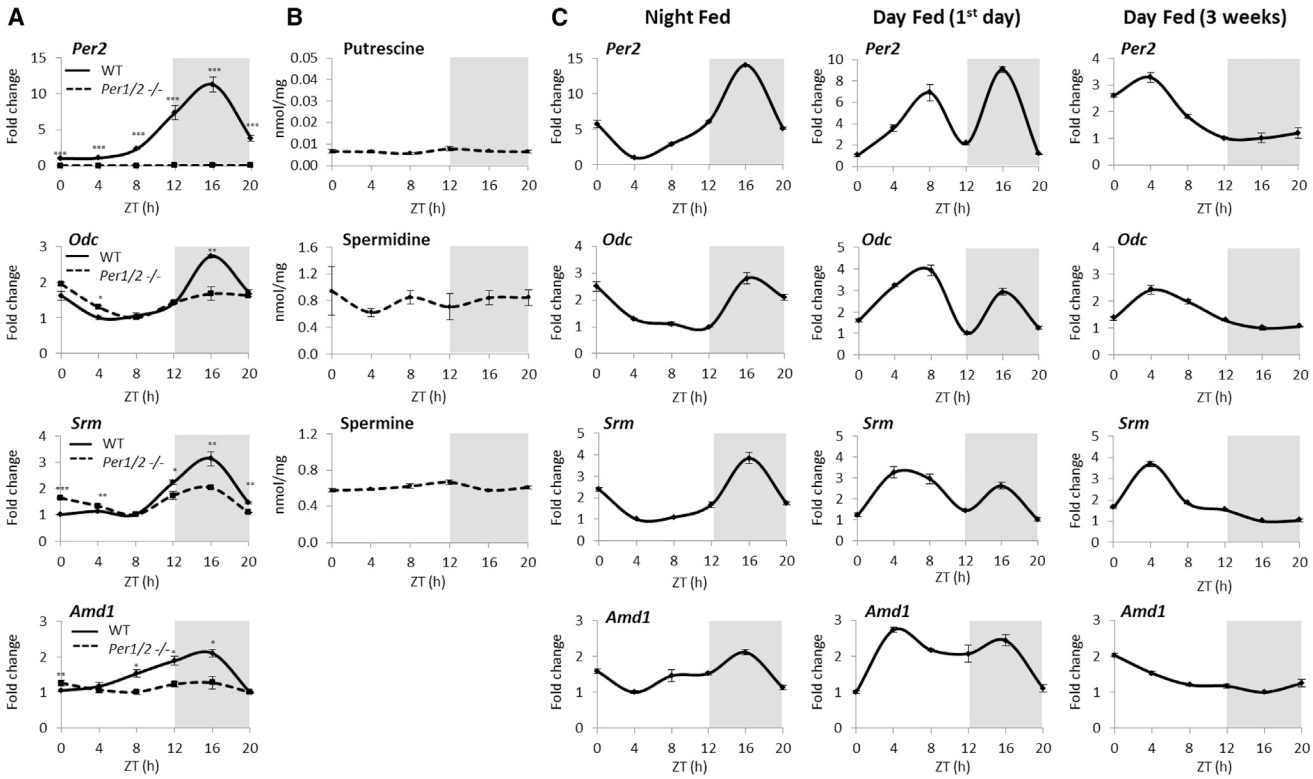

Figure 2. Clock- and Feeding-Dependent Mechanisms Regulate the Daily Expression of Key Enzymes in Polyamine Biosynthesis and Polyamine Accumulation

(A) Wild-type and *Per1/2* −/− mice were sacrificed at 4 hr intervals throughout the day. Total RNA was prepared from liver and transcript levels of *Per2*, *Odc*, *Srm*, and *Amd1* were determined by quantitative real-time PCR and presented as fold change relative to the lowest value.

(B) The levels of putrescine, spermidine, and spermine in livers of *Per1/2* −/− mice were quantified by HPLC and presented in nmol/mg tissue with mean ± SEM of 4 mice per time point.

(C) Wild-type mice were kept under 12 hr light-12 hr dark regimen and were fed exclusively during the night for 1 week, and subsequently the feeding regimen was inverted to daytime feeding for 3 weeks. Mice were sacrificed at 4 hr intervals throughout the day upon nighttime feeding (Night Fed), daytime feeding during the first day (Day Fed, 1st day), and daytime feeding following 3 weeks (Day Fed, 3 weeks). Total RNA was prepared from liver and transcript levels of *Per2*, *Odc*, *Srm*, and *Amd1* were determined by quantitative real-time PCR and presented as fold change relative to the lowest value. Expression data are presented as mean ± SD of 4 mice per time point. *p < 0.05, **p < 0.01, ***p < 0.001. Gray shading represents the dark phase; zeitgeber time, ZT. See also Figure S2.

Disruption of Polyamine Homeostasis Affects Circadian Rhythmicity

The daily regulation of polyamine biosynthesis prompted us to examine whether polyamines might in turn play a role in circadian rhythmicity. To maintain their polyamine levels within a narrow range, cells tightly regulate the influx of polyamines from the extracellular milieu (Casero and Marton, 2007). Indeed, polyamine supplementation (e.g., spermidine) to the growth medium of NIH 3T3 stably expressing a luciferase reporter gene under the control of the *Per2* promoter (*Per2*-luciferase reporter) neither affected their intracellular polyamine levels nor significantly altered their circadian oscillations (Figures S4A–S4C). Hence, to attain elevated levels of intracellular polyamines and examine their effect on circadian rhythmicity, we employed NIH 3T3 cells stably overexpressing ODC (NIH 3T3-ODC) and NIH 3T3 cells overexpressing AzI (NIH 3T3-AzI), which inhibits ODC degradation. Cells were transduced with a Lenti-virus carrying the *Per2*-luciferase reporter. Both NIH 3T3-ODC and NIH 3T3-AzI exhibited poor circadian oscillations compared to NIH 3T3 mock transfected cells (Figure 4A). SDS-PAGE and immunoblot analysis confirmed the elevated levels of ODC and AzI protein in NIH 3T3-ODC cells and NIH 3T3-AzI cells, respectively (Figure 4B), and polyamine levels were elevated in these cells (Figure 4C). These results infer that polyamine homeostasis is critical for the clock's function.

Next, we performed the reciprocal experiment in which we examined whether polyamine depletion affects circadian oscillations in cultured cells. To substantially deplete cellular polyamines, we pretreated cells with α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC enzymatic activity (Pegg and Casero, 2011). An equal number of DFMO-treated and non-treated cells were seeded and monitored for their circadian rhythmicity and polyamine content. Bioluminescence recordings of the *Per2*-luciferase reporter revealed that DFMO-treated cells exhibit an ~2 hr longer period (Figures 4D and 4E). As expected, polyamine levels were significantly reduced in DFMO-treated cells (Figure 4F). Similar results were obtained with NIH 3T3 cells stably expressing a luciferase reporter gene under the control of the *Bmal1* promoter (*Bmal1*-luciferase reporter), (Figures S4D–S4F).

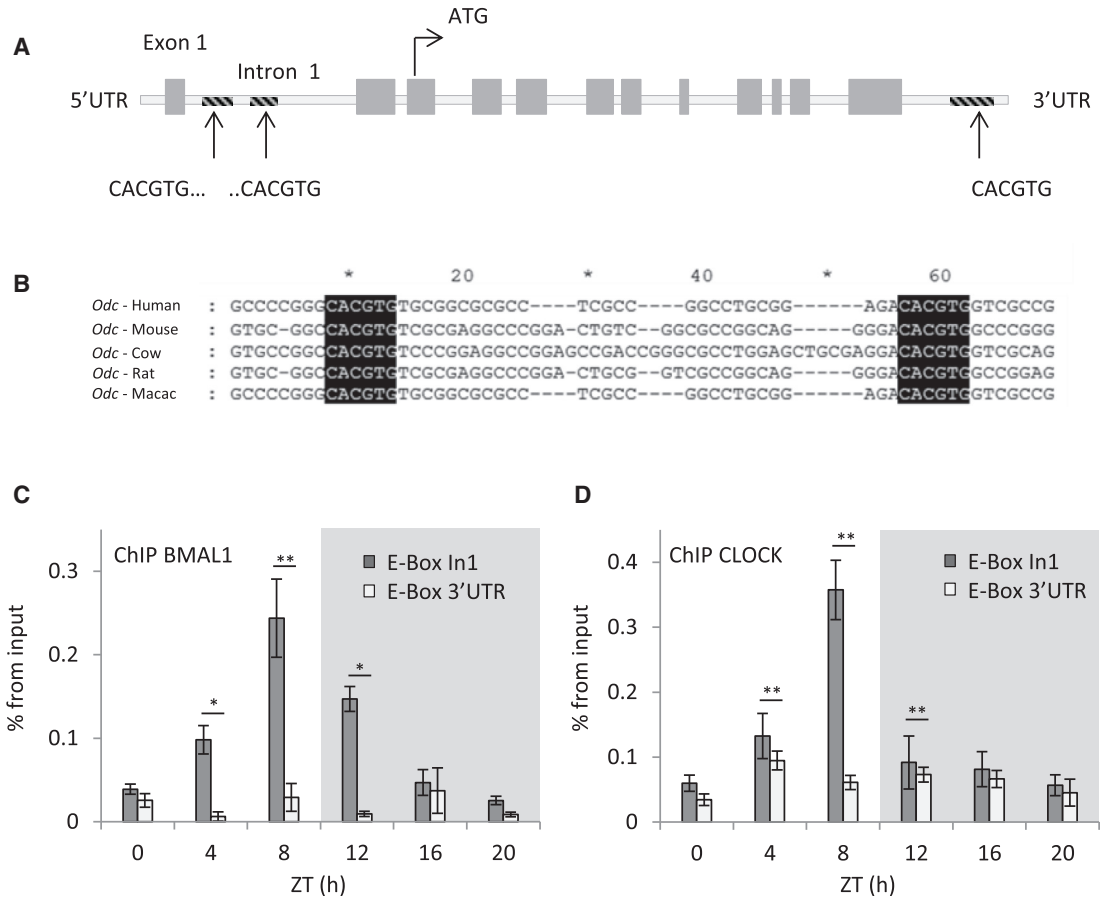

Figure 3. BMAL1:CLOCK Heterodimers Bind to E-Box Motif Present within the *Odc* Gene in a Daily Manner

(A) A schematic representation of the *Odc* gene. The E-box motifs (CACGTG) are indicated by black arrows.

(B) The tandem E-boxes sequence in intron 1 of *Odc* is conserved in mammals.

(C and D) Chromatin immunoprecipitation of BMAL1 (C) and CLOCK (D) with chromatin prepared from mice sacrificed at 4 hr intervals throughout the day. Real-time PCR was performed with primers specifically designed for E-boxes in *Odc* intron 1 (E-Box In1) and *Odc* 3′ UTR (E-Box 3′ UTR). Data are presented as percentage from input with mean ± SD of 3 mice per time point. *$p < 0.05$, **$p < 0.01$. Gray shading represents the dark phase; zeitgeber time, ZT. See also Figure S3.

To confirm that the effect of DFMO on the circadian period is due to polyamine-depletion, we supplemented DFMO-treated NIH 3T3 *Per2*-luciferase cells with ornithine, the precursor for polyamine biosynthesis, or with different polyamines. As predicted, addition of ornithine did not restore the circadian period, as ODC that converts ornithine to putrescine is inhibited by DFMO (Figures 4D and 4E). By contrast, addition of putrescine or spermidine, which bypasses ODC decarboxylase activity, was sufficient to restore the circadian period as in non-treated cells (Figures 4D and 4E). Concurrently, measurements of cellular polyamine content demonstrated the efficient uptake of polyamines by the depleted cells and the inter-conversion of the different polyamine species (Figure 4F). Similar results were obtained with primary tail fibroblast prepared from mice in which full-length PER2 protein was fused to LUCIFERASE (Figures S4G–S4I).

To further examine the effect of polyamine depletion on circadian oscillations, we employed genetic approaches to downregulate ODC expression in cells, either by overexpression of Az, which targets ODC for degradation, or by knockdown of ODC. NIH 3T3 cells were transfected with the *Per2*-luciferase reporter together with an Az expression vector or an empty vector. Az overexpression was validated by SDS-PAGE and immunoblot (Figure 4I). The decrease in spermidine levels upon Az overexpression (Figure 4J) was accompanied by lengthening of the circadian period (Figures 4G and 4H). Similarly, knockdown of ODC, using *Odc*-specific siRNA in NIH 3T3 *Bmal1*-luciferase cells (Figure 4M), decreased cellular polyamine levels (Figure 4N) and resulted in a longer circadian period (Figures 4K and 4L).

We conducted a set of control experiments to rule out the possibility that polyamine depletion affect the circadian period due to broad non-specific effects. First, DFMO treatment did not affect cell viability (Figure S5A) or the recordings of a luciferase reporter gene under the control of a CMV promoter (CMV-luciferase), (Figures S5B and S5C) excluding toxic or pleotropic effects. Second, in contrast to spermidine, neither K^+, Zn^{2+}, nor Mg^{2+} restored the circadian period of DFMO-treated NIH 3T3 *Per2*-luciferase cells, excluding the possibility that polyamines

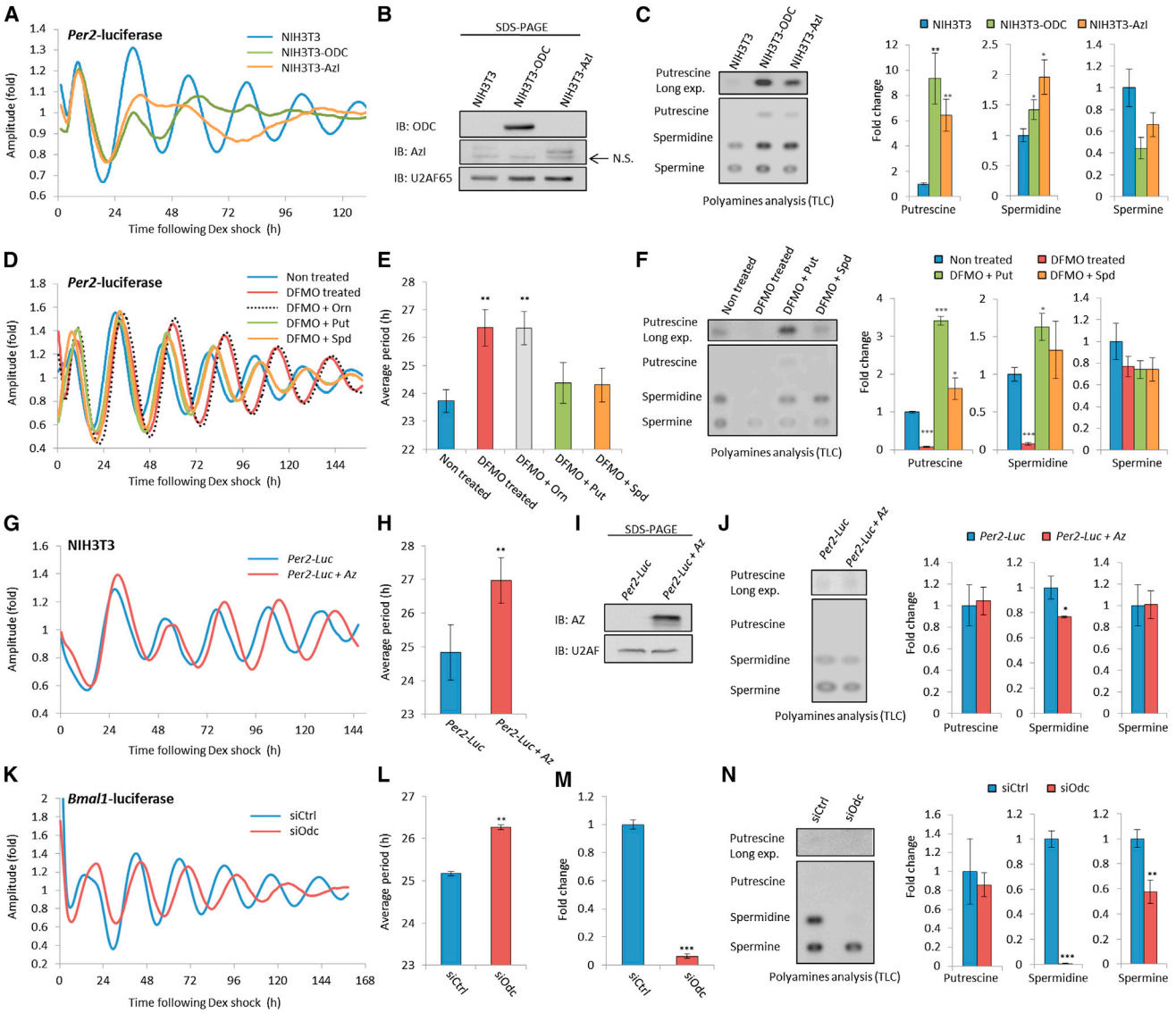

Figure 4. Disruption of Polyamine Homeostasis Affects Circadian Rhythmicity

(A) Bioluminescence recordings of NIH 3T3 mock-transfected, NIH 3T3 overexpressing ODC (NIH 3T3-ODC), and NIH 3T3 overexpressing Azl (NIH 3T3-Azl) stably expressing *Per2*-luciferase reporter.

(B) Protein extracts were prepared from NIH 3T3, NIH 3T3-ODC, and NIH 3T3-Azl and analyzed by SDS-PAGE and immunoblot (IB).

(C) Cellular polyamine content of NIH 3T3, NIH 3T3-ODC, and NIH 3T3-Azl was analyzed by thin-layer chromatography (TLC). The intensity of the different polyamine species was quantified and presented as fold change.

(D) Bioluminescence recordings of NIH 3T3 *Per2*-luciferase cells untreated, DFMO-treated, or DFMO-treated and supplemented with 100 μM ornithine (Orn), putrescine (Put), or spermidine (Spd).

(E) Bar graph representation of the average period length of the experiment described in (D).

(F) Cellular polyamine content under the different treatments described in (D).

(G) Bioluminescence recordings of NIH 3T3 cells that were co-transfected either with *Per2*-luciferase and an empty vector (*Per2*-luc) or with antizyme expression vector (*Per2*-luc+Az).

(H) Bar graph representation of the average period length of NIH 3T3 cells expressing *Per2*-luc and *Per2*-luc+Az.

(I) Protein extracts were prepared from NIH 3T3 cells expressing *Per2*-luc and *Per2*-luc+Az and analyzed by SDS-PAGE and immunoblot (IB).

(J) Cellular polyamine content of NIH 3T3 cells expressing *Per2*-luc and *Per2*-luc+Az.

(K) Bioluminescence recordings of NIH 3T3 *Bmal1*-luciferase cells that were transfected either with siRNA control (siCtrl) or siRNA targeting *Odc* (siOdc).

(L) Bar graph representation of the average period length of NIH 3T3 *Bmal1*-luciferase siCtrl and siOdc cells.

(M) Total RNA was prepared from NIH 3T3 *Bmal1*-luciferase siCtrl and siOdc cells and *Odc* transcript levels were determined by quantitative real-time PCR and presented as fold change relative to the highest value.

(N) Cellular polyamine content of NIH 3T3 *Bmal1*-luciferase siCtrl and siOdc cells. Data are presented as mean ± SD of three individual experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N.S., non-specific. See also Figures S4 and S5.

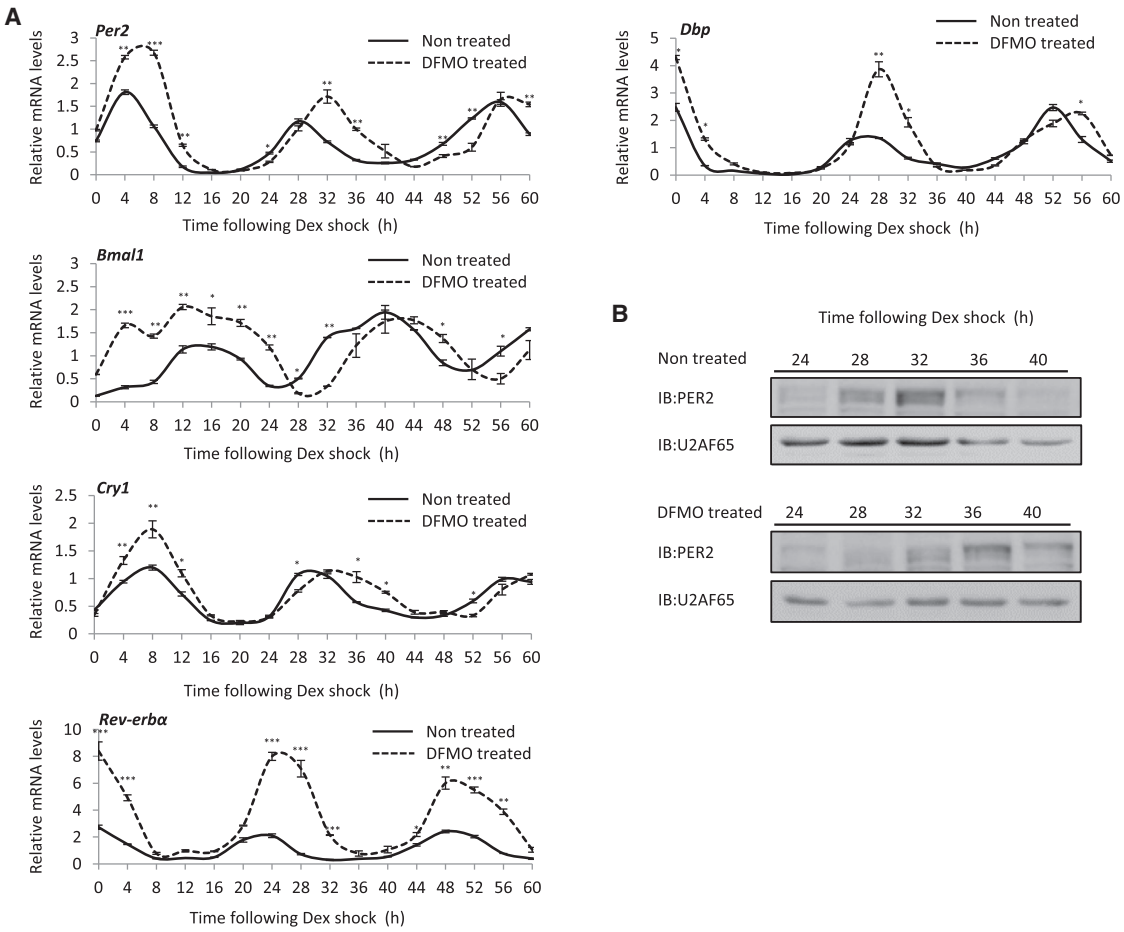

Figure 5. Polyamine Depletion Alters Endogenous Circadian Gene Expression and Protein Accumulation

(A) NIH 3T3 untreated and DFMO-treated cells were synchronized with a Dexamethasone (Dex) shock and harvested at 4 hr intervals throughout 60 hr. Total RNA was prepared and mRNA expression levels were determined by quantitative real-time PCR and presented as relative expression to an housekeeping gene. Data are presented as mean ± SD of 3 individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Protein extracts were prepared from NIH 3T3 and DFMO-treated cells and analyzed by SDS-PAGE and immunoblot (IB).

affect circadian oscillations due to their ionic charge (Figures S5D and S5E). Third, we examined whether other intermediate metabolites that are related to ornithine metabolism such as arginine, glutamine, or even GABA might be implicated in the effect of polyamine depletion on the circadian period. Unlike spermidine, none of these metabolites restored the circadian period length of DFMO-treated NIH 3T3 *Per2*-luciferase cells (Figures S5F and S5G).

Taken together, based on pharmacological and genetic manipulations of polyamine levels in cultured cells, we concluded that low polyamine levels lengthen the circadian period, while highly elevated cellular polyamine levels impair circadian rhythmicity.

Polyamine Depletion Affects Endogenous Circadian Gene Expression and Protein Accumulation

To corroborate the above-described findings, we monitored mRNA and protein expression levels of endogenous clock genes in untreated and polyamine depleted cells at 4 hr intervals for 2 consecutive days. In agreement with the data obtained using the different circadian reporters, the mRNA accumulation profile of several clock genes (i.e., *Per2*, *Bmal1*, *Cry1*, *Rev erbα*) and output gene (i.e., *Dbp*) exhibited a longer period with delayed second and third peaks upon DFMO treatment (Figure 5A). We also observed more than 3-fold increase in the amplitude of the oscillations of *Rev erbα* and *Dbp* upon polyamine depletion, whereas the amplitude of *Per2*, *Bmal1*, and *Cry1* oscillations was unaffected. In accordance with *Per2* mRNA expression, the peak in PER2 protein accumulation was also delayed upon polyamine depletion (Figure 5B). Hence, polyamine depletion lengthened the circadian period of cycling endogenous core clock and output genes.

Polyamines Modulate the Interaction between PER2 and CRY1

We wished to examine the molecular mechanisms through which polyamines exert their effect on circadian rhythmicity. In view of their role in regulation of protein-protein interactions, we set out to test the effect of polyamines on the interactions between clock proteins in living cells. We centered our analysis on

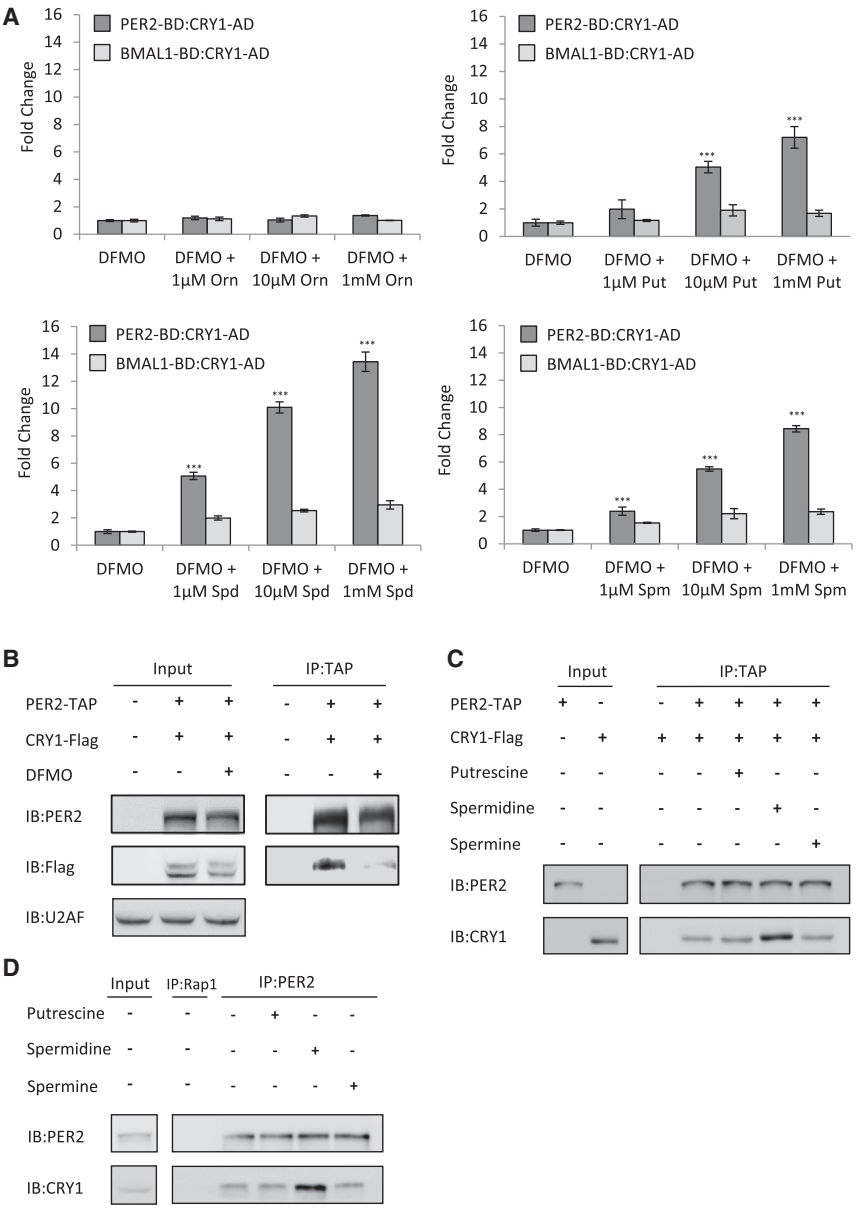

Figure 6. Polyamines Modulate the Interaction between the Core Clock Proteins

(A) PER2-BD together with CRY1-AD and Gal4-luciferase or BMAL1-BD together with CRY1-AD and Gal4-luciferase were expressed in NIH 3T3 cells; DFMO-treated cells were supplemented without or with 1 μM, 10 μM, or 1 mM ornithine (Orn), putrescine (Put), spermidine (Spd), or spermine (Spm) and bioluminescence was quantified 15 hr following a dexamethasone shock. Data are presented as mean ± SD of 3 individual experiments.

(B) DFMO-treated or untreated 293HEK cells were transfected without or with PER2-TAP and CRY1-Flag expression vectors. PER2-TAP was immunoprecipitated (IP) and the immunoprecipitated proteins were analyzed by immunoblot (IB) with Flag and PER2 antibodies.

(C) DFMO-treated NIH 3T3 cells were transfected with PER2-TAP or CRY1-Flag expression vectors. Protein extracts were prepared and mixed together in the absence or presence 10 μM putrescine, spermidine, or spermine. PER2-TAP was immunoprecipitated (IP) and the immunoprecipitated proteins were analyzed by immunoblot (IB) with PER2 and CRY1 antibodies.

(D) Liver nuclear extracts were prepared from mice fed diet low in polyamines together with DFMO. PER2 was immunoprecipitated (IP) in the absence or presence 10 μM putrescine, spermidine, or spermine and the immunoprecipitated proteins were analyzed by immunoblot (IB) with PER2 and CRY1 antibody. Rap1 antibody was used as a negative control. See also Figure S6.

two principal interactions within the core clock circuitry, namely BMAL1:CRY1 and PER2:CRY1, which participate in circadian period control. The former addresses the interaction between a repressor and an activator and the latter the binding within the repressor complex. To this aim, we employed a mammalian two-hybrid assay, in which BMAL1 or PER2 are fused to a DNA binding domain (BMAL1-BD and PER2-BD, respectively) and CRY1 to a transactivation domain (CRY1-AD). Binding of CRY1 to either BMAL1 or PER2 drives the expression of a Gal4-luciferase reporter and is detected by an increase in bioluminescence emitted from the cells (Langmesser et al., 2008). When polyamine-depleted NIH 3T3 cells were supplemented with putrescine, spermidine, or spermine, the interaction between PER2:CRY1 was increased (Figure 6A). The effect was dose dependent within a physiological concentration range (Casero and Marton, 2007) and specific to PER2:CRY1, as little effect on BMAL1:CRY1 interaction was observed (Figure 6A). Already concentrations as low as 1 μM of spermidine induced a considerable increase in PER2:CRY1 binding. As expected, addition of ornithine to DFMO-treated cells could not circumvent the inhibition of ODC-activity and as such did not enhance PER2:CRY1 interaction (Figure 6A). Taken together, these experiments suggested that polyamines specifically promote the interaction between PER2 and CRY1 in living cells with spermidine being the most potent.

To further delineate the effect of polyamines on PER2:CRY1 interaction, we performed co-immunprecipitation experiments from cultured cells. PER2 and CRY1 were expressed in untreated or DFMO-treated 293HEK cells. In line with the above described experiments, PER2:CRY1 binding was reduced in polyamine-depleted cells (Figure 6B). Similar results were obtained with endogenous PER2 and CRY1 in untreated and polyamine-depleted NIH 3T3 cells throughout the circadian cycle (Figure S6A).

To examine whether polyamine can regulate the binding in vitro, we expressed PER2 and CRY1 in DFMO-treated cells; cell extracts were prepared and mixed in the absence or presence of putrescine, spermidine, or spermine. Co-immunoprecipitatation experiments showed that spermidine promotes

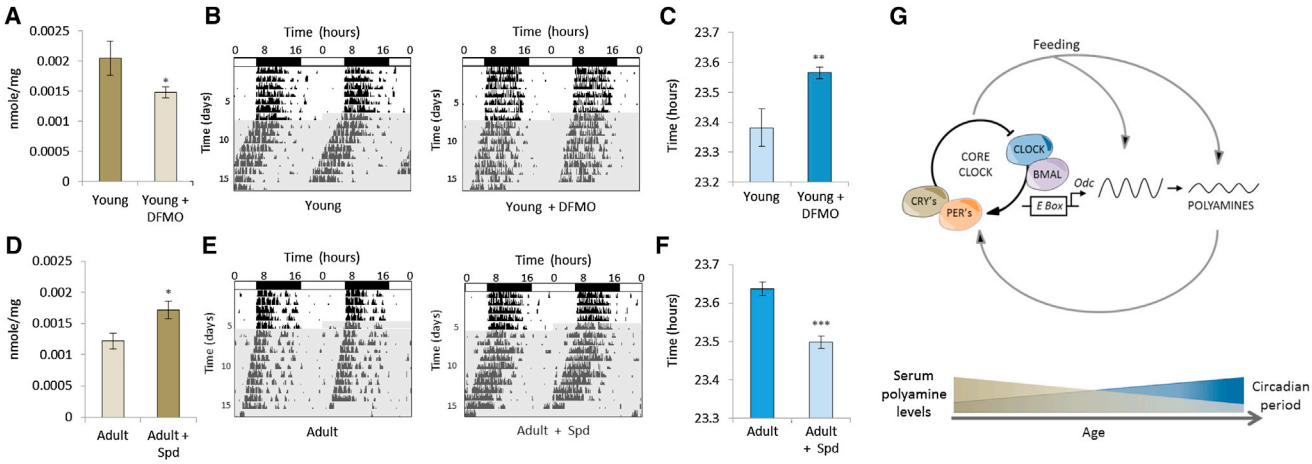

Figure 7. Polyamine Levels Modulate the Circadian Period in Mice

(A–C) 3-month-old mice were fed low polyamine diet to minimize polyamine uptake from the food and were provided without (young) or with 2% DFMO (young + DFMO) in their drinking water for 1 month. (A) Quantification of serum spermidine levels (ZT16). Data are presented in nmol/mg protein with mean ± SEM of 5 mice. (B) Representative double-plot actograms for the wheel-running locomotor activity of young and young + DFMO mice. In each actogram, the first few days were recorded under 12 hr light-12 hr dark conditions, after which the light was turned off and recording was continued in constant darkness. Time spans during which the lights were switched off are marked by gray shading. (C) Period lengths of young and young + DFMO mice in constant darkness. Free-running period lengths in hours were 23.38 ± 0.06 (young) and 23.56 ± 0.01 (young + DFMO). The bar diagram represents the mean ± SEM of 10 mice, Student's t test with p value = 0.0082.

(D–F) 8-month-old mice were provided without (adult) or with 5 mM spermidine (adult + Spd) in their drinking water for 5 months. (D) Quantification of serum spermidine levels (ZT16). Data are presented in nmol/mg protein with mean ± SEM of 9 mice. (E) Representative double-plot actograms for the wheel-running locomotor activity of adult and adult + Spd mice. (F) Period lengths of adult and adult + Spd mice in constant darkness. Free-running period lengths in hours were 23.63 ± 0.01 (adult) and 23.49 ± 0.01 (adult + Spd). The bar diagram represents the mean ± SEM of 10 mice, Student's t test with p value = 1.59E-05.

(G) A schematic model depicting the metabolic feedback loop between circadian clocks and polyamine biosynthesis that participates in circadian period control. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S7.

PER2:CRY1 binding both in NIH 3T3 and 293HEK cells (Figures 6C and S6B, respectively). Similar experiments were performed with liver nuclear extracts prepared from mice fed diet low in polyamines together with DFMO. Here again, PER2:CRY1 interaction was augmented in the presence of spermidine (Figure 6D).

To specifically dissect the effect of polyamines on PER2, we performed a partial trypsin digestion assay with in vitro translated [35S]-labeled proteins (Figure S6C). The trypsin digestion of only PER2 but not of PER1, CRY1, REV-ERBα, or BMAL1 was altered exclusively in the presence of spermidine and not of putrescine or spermine, supporting the possibility that spermidine specifically binds to PER2 and imposes a conformational change.

In conclusion, our experiments evinced that polyamines, most likely spermidine, regulate PER2:CRY1 interaction, plausibly by inflicting a conformational change on PER2. It is conceivable that the effect of polyamines on PER2:CRY1 binding serves as a mechanistic basis for the circadian clock control by polyamines.

Polyamine Levels Modulate the Circadian Period in Mice

Our experiments in cultured cells revealed that polyamines participate in circadian period control and prompted us to examine whether these findings are also physiologically relevant in mice. Since *Odc* knockout mice are non-viable (Pendeville et al., 2001), we centered our efforts on pharmacological/nutritional modulation of polyamine levels. First, we quantified spermidine levels in young (4 months) and adult mice (13 months) and found in line with previous reports (Nishimura et al., 2006) that polyamine levels decrease with age (Figure S7A). Notably, the circadian period of locomotor activity under free running conditions, in constant darkness, was longer in adult mice compared to young mice (4 months old 23.41 ± 0.05; 13 months old 23.63 ± 0.01; p value = 0.0034), (Figures S7B and S7C) (Valentinuzzi et al., 1997). Thus, low polyamine levels correlated with longer circadian period, in agreement with the results obtained in cultured cells (Figures 4 and 5).

To examine causality, we asked whether the circadian period in young mice is lengthened upon decrease in polyamine levels, and conversely whether increase in polyamine levels shortens the circadian period in adult mice. To this aim, young mice received a diet low in polyamines (Atiya Ali et al., 2011) together with DFMO in their drinking water to decrease their polyamine levels. On the other hand, adult mice were provided with spermidine in their drinking water to increase polyamine levels. As expected, upon DFMO treatment the serum levels of spermidine were reduced by ~30% (Figure 7A). Under free-running conditions, DFMO-treated mice exhibited a statistically significant longer circadian period (young 23.38 ± 0.06; DFMO-treated young mice 23.56 ± 0.01; p value = 0.0082) (Figures 7B and 7C). It should be noted that unlike in cultured cells in which DFMO strongly depleted polyamine levels, in mice the effect was much more moderate, as mice can partially replenish their polyamine levels from the food even when they are on a low polyamine diet.

In the reciprocal experiment, supplementation of spermidine in the drinking water to adult mice resulted in ~35% increase

in spermidine serum levels (Figure 7D). Spermidine-treated mice exhibited a statistically significant shorter circadian period under free-running conditions (adult 23.63 ± 0.01; spermidine treated adult mice 23.49 ± 0.01; p value = 0.59E-05) (Figures 7E and 7F).

Taken together, our results suggested, similar to our observations in cultured cells, that polyamine levels control the circadian period in mice. Low levels of polyamine lengthen the circadian period, while elevated levels shorten it. Thus, it is conceivable that the decline in polyamine levels with age might play a role in circadian period lengthening that is associated with aging. Furthermore, we demonstrated that nutritional interventions could be applied to modulate polyamine levels and consequently alter the circadian period.

DISCUSSION

The crosstalk between circadian clocks and metabolism raises the intriguing possibility that metabolites and metabolic pathways that are temporally gated by the clock play in turn a prominent role in the clock's function. Such a feedback loop mechanism might serve to fine-tune and reinforce the function of clock. Hitherto, the best-documented example for such a scenario is NAD^+. Circadian clocks regulate the daily oscillations in NAD^+ levels, in turn NAD^+ levels regulate the clock's function (Asher and Sassone-Corsi, 2015) through a mechanism that decays with aging (Chang and Guarente, 2013). In this study, we provide evidence that a metabolic feedback loop applies for a complete different group of metabolites, namely polyamines, and participates in circadian period control (Figure 7G).

We show that *Odc*, *Srm*, and *Amd1* and the products of their enzymatic activity, putrescine and spermidine, oscillate in liver in a daily manner. These oscillations are blunted in *Per1/2* null mice. Notably, circadian expression datasets of various clock mutant mice revealed that *Odc*, *Srm*, and *Amd1* are also nonrhythmic in the liver of *Cry1/Cry2* null mice (Vollmers et al., 2009) and of *Clock* mutant mice (Miller et al., 2007). Moreover, we demonstrate that BMAL1:CLOCK bind in a daily manner to conserved E-box elements within the *Odc* gene. Along this line, genome-wide analysis of DNA-binding sites for BMAL1:CLOCK identified binding sites also on *Srm* (Koike et al., 2012). Recently, microarray expression profiling on cultured hepatocytes uncovered circadian oscillations in gene expression of multiple metabolic pathways, specifically *Odc* and *Srm* displayed cell-autonomous shallow circadian oscillations (Atwood et al., 2011). Taken together, these findings further support our conclusions that key polyamine metabolic enzymes are under circadian clock control.

Feeding-dependent mechanisms also appear to play a role in the daily oscillations of polyamine metabolism. This can be achieved through expression regulation of polyamine metabolic enzymes by feeding cycles and via uptake of polyamines from the diet. Notably, in addition to nutritional supply of polyamines directly from the diet, microorganisms in the gut are also capable of synthesizing polyamines (Matsumoto et al., 2012). Recent studies demonstrated that feeding schedule influences the composition of gut microbiota (Thaiss et al., 2014; Zarrinpar et al., 2014). Thus, feeding cycles can also support oscillations in polyamine levels indirectly through coordination of microbiota population in the gut that synthesizes polyamines. The serum polyamine levels are likely to reflect the integration of the aforementioned homeostatic mechanism.

Our model suggests that polyamine levels control the circadian period through regulation of PER2:CRY1 interaction. Since the different polyamine species (i.e., putrescine, spermidine, or spermine) are readily interconverted within the cell, we cannot exclude the possibility that all three species participate in the process. Nevertheless, our analyses suggest that spermidine plays a prominent role as it exhibits the strongest effect on PER2:CRY1 interaction both in vivo and in vitro and specifically affects the trypsin digestion of PER2. Indeed, out of the different polyamine species, spermidine is considered to be the biologically most active form in various cellular processes (Pegg, 2009). Though we detect daily oscillations in hepatic polyamine levels, primarily with putrescine and to a lesser extent with spermidine, we cannot ascertain that these oscillations per se are indeed important for the clock's function. Notably, PER2:CRY1 binding peak ~ZT16 (Asher et al., 2008), which coincide with the zenith levels of putrescine and spermidine in liver.

It is possible that polyamines also affect the clock's function via additional mechanisms beside regulation of PER2:CRY1 interaction. Since polyamines are also known to participate in modulation of protein-DNA interactions, they might also regulate the binding of the clock transcription factors to their cognate DNA-binding sites. Future studies are expected to shed more light about the presence and nature of these additional mechanisms.

Recently, the crystal structure of the complex between the CRY1-photolyase homology region and a C-terminal PER2 fragment was determined. This structure revealed the presence of a jointly coordinated zinc ion and suggested that it stabilizes PER2:CRY1 interaction (Schmalen et al., 2014). Like zinc, spermidine is also positively charged, yet its positive charge is dispersed over several angstroms, inferring that spermidine binds the complex in a distinct manner from zinc. Along this line, unlike spermidine, addition of zinc failed to restore the circadian period in polyamine-depleted cells (Figures S5D and S5E).

The decline in polyamine levels with age is associated with lengthening of the circadian period. In agreement with previous reports, the difference between young and adult mice is several minutes (~14 min). We intentionally did not use old mice, to avoid confounding effects that are associated with old age. Under this physiological context we show that polyamine depletion lengthens the circadian period in young mice, whereas polyamine supplementation shortens it in adult mice. The effects are within the range of few minutes (~11 and ~9 min, respectively), yet even small deviations of the innate circadian period from that of the environment are associated with metabolic and age-related pathologies (Libert et al., 2012). One reason for the relative small effects compared to the one obtained in cultured cells is that mice are resilient to major changes in their polyamine levels and maintain them within a narrow physiological range through various homeostatic mechanisms. Indeed, even the combination of low polyamine diet together with DFMO resulted only in ~30% decrease in polyamine levels, most likely due to an increase in polyamine uptake from the diet. Likewise polyamine supplementation for weeks led to a moderate increase (~35%) in spermidine levels.

Previous studies mostly examined the effect of nutritional challenges such as high fat diet on circadian clocks and identified profound reorganization of the molecular clock and various outputs (Eckel-Mahan et al., 2013). Here we show that certain diets can be in fact beneficial for circadian rhythmicity and that supplementation of spermidine in the diet can maintain the circadian period in adult mice, similar to young mice. Remarkably, chronic spermidine administration has been shown to promote health span and extend lifespan (de Cabo et al., 2014); these effects might be mediated in part through the preventive effect of spermidine on clock's function with age.

In summary, we propose herein a metabolic feedback loop that couples circadian oscillators with polyamine biosynthesis (Figure 7G). Furthermore, our results open new possibilities for nutritional interventions that can modulate the circadian period. Thus, polyamine supplementation might serve as future strategies to treat age-dependent decline in clock's function.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments and procedures were conducted in conformity with the Institutional Animal Care and Use Committee (IACUC) guidelines. Unless indicated differently, 3-month-old male wild-type and *Per1/2* −/− mice (Zheng et al., 2001) were used. Mice were kept under 12 hr light-12 hr dark regimen and fed either ad libitum or exclusively during the dark or light phase as indicated. ZT0 corresponded to lights on and Z12 to lights off in the animal facility.

Monitoring Wheel Running Activity

Circadian locomotor activity was determined by monitoring wheel-running activity. The first days were recorded under 12 hr light-12 hr dark conditions, after which the light was turned off and recording was continued in constant darkness. The circadian period was analyzed with the ClockLab software (Actimetrics).

Cell Culture, Plasmids, and Transfections

NIH 3T3, NIH 3T3-ODC, NIH 3T3-Azl (Keren-Paz et al., 2006), NIH 3T3 *Bmal1*-luciferase (Nagoshi et al., 2004), tail fibroblasts from PER2-LUCIFERASE fusion knockin mice (Yoo et al., 2004) and 293HEK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and cultured at 37°C in a humidified incubator with 5% CO_2. To generate stable cell lines expressing the *Per2*-luciferase reporter, we transduced NIH 3T3, NIH 3T3-ODC, and NIH 3T3-Azl with a Lenti-virus carrying the *Per2*-luciferase reporter as previously described (Liu et al., 2008). The following plasmids were used: pEF-IRES, pEF-IRES-Az, pCDNA PER2-Flag, pCDNA PER1-Flag, pCDNA CRY1-Flag, pCDNA Flag-REV-ERBα, pCDNA BMAL1-Flag, pCMV LUC-TAP, pCMV PER2-TAP, and for the mammalian two-hybrid assay; pFR-Gal4-luciferase and GAL4-PER2 (PER2-BD) (kindly provided by U. Schibler), GAL4-BMAL1 (BMAL1-BD), and CRY1-VP16 (CRY1-AD), (kindly provided by U. Albrecht). Cell transfections were carried out with Lipofectamine2000 transfection reagent. ON-TARGET plus mouse *Odc* siRNA and control siRNA (Thermo scientific) were delivered into the cells with Lipofectamine RNAi reagent.

RNA Preparation and Real-Time PCR Analysis

RNA extraction and transcript quantification by real-time PCR were carried out as previously described (Adamovich et al., 2014). Real-time PCR measurements were performed using SYBR green or Taqman probes with LightCycler II machine (Roche) and normalized to the housekeeping gene *Tbp*. Primers and probes are listed under Supplemental Experimental Procedures.

Real-Time Bioluminescence Monitoring

Cells were seeded in 35 mm culture dishes at ~80% confluence and synchronized with 100 nM dexamethasone treatment for 20 min. Bioluminescence was recorded using the LumiCycle apparatus and data were analyzed with the LumiCycle analysis software (Actimetrics).

Protein Extraction, Gel Electrophoresis, and Immunoblotting

Mouse liver protein nuclear extracts were prepared according to the NUN procedure as previously described (Shalev et al., 2014). Cultured fibroblasts were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris-Hcl [pH 8], 1 mM dithiothreitol) supplemented with protease inhibitors (1 mM N-(α-aminoethyl) benzene-sulfonyl fluoride, 40 μM bestatin, 15 μM E65, 20 μM leupeptin, 15 μM pepstatin; Sigma). The extracts were centrifuged to remove cell debris at 13,000 rpm for 10 min at 4°C. Samples were heated at 95°C for 5 min in Laemmli sample buffer and analyzed by SDS-PAGE and immunblot. Antibodies used were the following: rabbit anti-CRY1, PER2, Rap1 (Asher et al., 2010), mouse anti-Flag, ODC, Az, and U2AF65 (Sigma).

Co-immunoprecipitation Experiments

Experiments were carried out with mouse liver nuclear extracts or with cultured cells extracts as specified. Extracts were incubated for 2 hr with the indicated antibodies at room temperature and subsequently incubated with protein A/G agarose beads (Santa Cruze) for an additional 1 hr at room temperature. For immunoprecipitation of TAP tagged proteins, extracts were incubated with mouse IgG-Agarose beads (Sigma). Beads were washed with NP40 buffer (100 mM Tris-HCL [pH 7.5], 150 mM NaCl, 2 mM EDTA, and 1% NP40), and samples were mixed with Laemmli sample buffer and heated at 95°C for 5 min.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.09.011.

ACKNOWLEDGMENTS

We thank T. Tsimbalist, S. Meir, and I. Rogachev for the polyamines quantification and S. Ovadia for his help in bleeding the mice. We are grateful to U. Schibler for his valuable comments. The work performed in the G.A. laboratory was supported by the Israel Science Foundation (ISF 138/12), the Abish-Frenkel Foundation, the HFSP Career Development Award (HFSP CDA00014/2012), and the European Research Council (ERC-2011 METACYCLES 310320). G.A. and H.R. are funded by GIF collaborative grant (G-1199-230.9/2012).

Received: March 20, 2015
Revised: July 15, 2015
Accepted: September 9, 2015
Published: October 8, 2015

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