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MicroRNAs modulate core-clock gene expression in pancreatic islets during early postnatal life

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Abstract

Aims/hypothesis

Evidence continues to emerge detailing a fine-tuning of the regulation of metabolic processes and energy homeostasis by cell-autonomous circadian clocks. Pancreatic beta-cell functional maturation occurs after birth and implies transcriptional changes triggered by a shift in the nutritional supply occurring at weaning, enabling the adaptation of insulin secretion. So far, the developmental timing and the exact mechanisms involved in the initiation of the circadian clock in the growing pancreatic islets have never been addressed.

Methods

Circadian gene expression was measured by qPCR in islets of rats at different postnatal ages up to 3 months, and by *in vitro* bioluminescence recording in newborn (10-day-old) and adult (3-month-old) islets. The effect of the microRNAs, miR-17-5p and miR-29b-3p, on the expression of target circadian genes was assessed in newborn rat islets transfected with microRNA antisense or mimic oligonucleotides, and luciferase reporter assays were performed in the rat insulin-secreting cell line INS832/13 to determine a direct effect. The global regulatory network between microRNAs and circadian genes was computationally predicted.

Results

We found major differences in the rhythmicity and expression of core circadian genes throughout postnatal beta-cell maturation. Synchronization of the clock machinery in cultured rat islets revealed a delayed cell-autonomous rhythmicity in newborn compared to adult cells. Computational predictions unveiled the existence of a complex regulatory network linking the microRNAs displaying modifications in their expression profiles during postnatal beta-cell maturation and key core clock genes. In agreement with these computational predictions, we demonstrated that miR-17-5p and miR-29b-3p directly regulate circadian gene expression in the maturing islet cells of 10-day-old rats.

Conclusions/interpretation

These data show that the circadian clock is not fully operational in newborn islets and that microRNAs potently contribute to its regulation during postnatal beta-cell maturation. Defects in this process may have long-term consequences on circadian physiology and pancreatic islet function, favoring the manifestation of metabolic diseases such as diabetes.

Keywords

Circadian clock; microRNAs; pancreatic islets; postnatal maturation

Abbreviations

INS: Insulin-secreting beta-cell derived line

miR: microRNA

P10: 10-day-old rats

qRT-PCR: Quantitative Reverse-Transcription Polymerase Chain Reaction

UTR: Untranslated region

Introduction

Early postnatal life is a critical period for the development of the endocrine pancreas and involves the maturation of secretory and mitogenic processes. We and others demonstrated that immediately after birth, newborn beta-cells are still immature [1-3]. They lack the unique and essential feature of mature beta-cells, the capacity to secrete insulin in response to elevated concentrations of glucose. In contrast, amino acid-stimulated insulin release as well as insulin biosynthesis are already fully operational. At the same time, newborn beta-cells proliferate intensively within the expanding pancreas to achieve an appropriate beta-cell mass that will then be maintained throughout the adult life. We found that beta-cell maturation is associated with a major reprogramming in gene expression. This process is at least in part governed by microRNAs (miRNAs), a class of small non-coding RNAs that inhibit translation and/or stability of target mRNAs by pairing to their 3'-untranslated region (UTR) [1]. Indeed, we demonstrated that modifications in islet miRNA levels taking place during the postnatal period directly control the expression of key metabolic and cell cycle genes, driving the maturation of glucose-responsive adult beta-cells.

Rodent and human pancreatic beta-cells, as most cells in the body, possess self-sustained molecular clocks that coordinate metabolism timing throughout the day and modulate insulin secretion to maintain blood glucose homeostasis [4-7]. The circadian oscillators operative in beta-cells and other peripheral cells are synchronized by systemic signals (e.g. neural and endocrine signals, fasting/feeding and metabolic cycles) controlled by a central pacemaker in the suprachiasmatic nucleus of the brain. This master clock, itself synchronized by daylight, maintains phase coherence among the body cellular clocks [8-10]. The clock machinery has a strong influence on gene expression programs and orchestrates the timing of metabolic processes by regulating in a circadian manner about 10% of the transcripts [11, 12]. Cell-autonomous feedback loops repeated every 24 hours are generated by the activation of the transcription factors CLOCK and BMAL1 (also known as ARNTL) that trigger the expression of their own repressors, periods (PER1,2,3) and cryptochromes (CRY1,2). CLOCK and BMAL1 also drive the rhythmic expression of Nr1d genes (Rev-erbα, Rev-erbβ) [9]. Moreover, the expression of clock genes is regulated at the post-transcriptional level by multiple other factors, including miRNAs [13].

Studies of rhythmic gene expression based on the disruption of clock genes in liver, pancreas, muscle, and adipose tissue, suggest that peripheral clocks may play an important

role in optimizing metabolism by coordinating the function of key metabolic organs to nutrient availability and energy requirements [14-17]. Importantly, previous studies in humans and rodents favor the view that the synchronized rhythmicity of clock gene expression matures at rates that differ between tissues and is the result of specific changes occurring in the environmental conditions of each organ [18-21]. Indeed, in humans, plasmatic circadian variations of the glucocorticoid hormone appearing after 3 months of age [22] are responsible for the changes of the circadian gene expression phases in liver, kidney and heart [23]. Consistently, a gradual completion of clock gene rhythmic expression during postnatal life has been reported in rat liver within 30 days after birth [24].

However, the establishment of circadian rhythms during pancreatic beta-cell development and its underlying mechanisms have not been investigated yet. Hence, in this study we determined the expression levels and circadian oscillation profiles of several core-clock genes in maturating beta-cells. Moreover, the role of nutrition-driven islet miRNAs changes in modulating circadian clockwork during postnatal development was assessed. We found that the expression and rhythmic oscillations of circadian genes are not fully established in newborn rat islets, and that miR-17-5p and miR-29b-3p, which display expression changes throughout beta-cell maturation, directly control islet clock gene expression. The setup of clock rhythmic expression may reflect the adaptation of the organism to changes in nutritional supply, such as the shift from a fat-rich maternal milk to a high-carbohydrate diet occurring at weaning [1, 25].

Material and methods

Animals

Male and pregnant Sprague-Dawley rats were obtained from Janvier laboratories and were housed on a 12:12h light-dark cycle in a climate-controlled and pathogen-free facility. All procedures were performed in accordance with the Guide for the care and use of laboratory animals from the National Institutes of Health and were approved by the Swiss Research Councils and Veterinary Office (authorization VD2608.1). At birth, litters were adjusted to 10–12 pups per dam to standardize mother milk availability. Newborn males and females were nursed until sacrifice. At P21, the pups were weaned on a standard chow diet containing 18.5% crude proteins, 4.5% fat and 54% carbohydrates.

Cell culture, transfection and lentiviral transduction

Pancreatic islets were isolated as described [26] by collagenase digestion followed by purification on Histopaque (Sigma-Aldrich, St-Louis, MO, USA) density gradients. The islets were cultured in RPMI 1640 Glutamax medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Amimed, London, UK), 50 U/ml penicillin, 50ug/ml streptomycin, 1 mmol/l Na Pyruvate and 250 µmol/l Hepes. The rat insulin-secreting cell line INS832/13 was cultured as described [27]. INS832/13 cells and dissociated rat islet cells were transfected using Lipofectamine 2000 (Invitrogen, Carslbad, CA, USA) [27]. To reduce miR-17-5p expression, ≈250,000 cells were transfected with a specific single-stranded anti-miR (miScript miRNA inhibitor; Qiagen, Hilden, Germany), or with the miScript miRNA reference inhibitor (Qiagen, Hilden, Germany) as a negative control. Overexpression of miR-17-5p and miR-29b-3p was achieved by transfecting cells with oligonucleotide duplexes (Eurogentec, Liege, Belgium and Qiagen, Hilden, Germany) using Lipofectamine 2000 (Invitrogen). A siRNA directed against GFP was used as negative control. The survival rate was > 90% in transfected cells. To monitor circadian bioluminescence, a *Bmal1*-luciferase (*Bmal1*-luc) construct [28] was introduced in ≈150,000 adherent cells by lentiviral transduction. To produce lentiviral particles, *Bmal1*-luc lentivectors were transfected into 293T cells using the polyethylenimine method [5, 29]. Dissociated rat islet cells were transduced twice, with a multiplicity of infection (MOI) = 3 for each transduction.

Measurement of miRNA and mRNA expression

Total RNA was extracted with the miRNeasy kit (Qiagen, Hilden, Germany). Mature miRNA and mRNA levels were assessed by qRT-PCR using the miScript II RT kit (Qiagen, Hilden, Germany). miScript primer assays and primer sequences are provided in Supplementary Table 1. miRNA expression was normalized to the level of U6. mRNA expression was normalized to the amount of 18S. Pool of pancreatic islets from P1 (4 pups), P5 (4 pups) and P10 (2 pups) were collected while islets from P15, P20, P23, P31 and adult rats were extracted individually.

Luciferase assays

Luciferase reporter plasmids were generated by cloning about 200 nucleotides of the 3'UTR of rat *Clock*, *Npas2* and *Per3*, containing the putative binding sites of miR-17-5p or miR-29b-3p into the psiCHECK-1 vector (Promega, Madison, WI, USA). Luciferase activity was measured in ≈180,000 INS832/13 cells with a dual-luciferase reporter assay (Promega, Madison, WI, USA) two days after transfection. Renilla luciferase activity from the psiCHECK-1 constructs was normalized for transfection efficiency to the SV40-driven Firefly activity generated from a co-transfected PGL3 promoter vector (Promega, Madison, WI, USA).

***In vitro* synchronization and circadian bioluminescence measurement**

Adherent transduced islet cells were synchronized by a 1h forskolin pulse (10µM; Sigma, Saint-Louis, MO, USA), and subjected to continuous bioluminescence recording in RPMI medium containing 100µM luciferin (D-luciferin 306-250, NanoLight Technology) as described [5]. Photon counts of each well were integrated during 1 min, over 24 min intervals. For detrended time series, raw luminescence signals were smoothened by a moving average with a window of 24h, as already described [7]. Average circadian amplitude and the period length were calculated based on five consecutive peaks of detrended profiles starting from the second circadian cycle. Average timing of positive peak phase was quantified starting from the second peak, relative to 24h day cycle [30].

Modelling of the core-clock mRNA-miRNA network during beta-cell maturation

We selected 62 miRNAs that are differentially expressed between newborn and adult rats [1]. For each miRNA of interest, we retrieved the target genes from several sources (DIANA-microT, ElMMo, miRBase, miRanda, miRDB, PicTar, PITA and TargetScan) using the *MultiMir* R package [31]. The retrieved interactions were filtered using the list of core clock

genes investigated in this study. The resulting network was visualized with Cytoscape [32]. The information about the expression changes between the islets of newborn and adult rats mapped on the network was inferred from the microarray data for the miRNAs [1] and the qPCR data (present study) for the core clock genes. The color of the edges of the network indicates the sign of the log fold change of each connected node. Anti-correlated interactions, suggesting a functional link between the miRNA and the mRNA, are highlighted.

Statistical analysis

Statistical differences were tested by using a Student's t-test for unpaired comparisons or with ANOVA followed by a post-hoc Dunnett test for multiple comparisons, with a discriminating p-value of 0.05 (Prism GraphPad). The amplitude differences between the consecutive circadian cycles in each profile were assessed using one-way ANOVA test.

Results

Rhythmic transcriptional oscillation of circadian genes is not yet established in newborn islets.

After birth, rat pancreatic beta-cells are unable to secrete insulin specifically in response to glucose and undergo a postnatal maturation process resulting in the acquisition of a functional adult beta-cell mass [1]. Since islets possess self-sustained molecular clocks that optimize cellular functions, including insulin secretion, we determined whether the islet circadian rhythmicity and its machinery differs between newborn and adult islets. For this purpose, we assessed the mRNA levels of several core circadian genes in newborn and adult rat islets across 24h (Fig.1). Interestingly, we found that the transcriptional oscillations of *Npas2*, *Bmal1*, *Rev-Erbα*, *Per1*, *Per2*, *Per3* and *Cry2* over 24h are strikingly attenuated in 10-day-old (P10) compared to 3-month-old adult islets (Fig.1b, c, e, f, g, h, j).

These findings can be explained either by an intrinsic incapacity of the genes to oscillate in neonatal islets or by the lack of synchronization between newborn cells. To investigate the cell-autonomous properties of the circadian clocks in newborn islets, the oscillation profile of the bioluminescent circadian reporter *Bmal1*-luc was recorded in cultured P10 and adult islet cells, following *in vitro* synchronization. When synchronized by forskolin, both P10 and adult islet cells exhibited self-sustained circadian oscillations of *Bmal1*-luc expression (Fig.2a, b), however the characteristics of these profiles were distinct (Fig.2c-e). Indeed, newborn islet cells displayed earlier first peak (Fig.2c; 5.51 ± 1.31h in P10 compared to 10.10 ± 0.74h in adults), and a circadian phase delay of almost 4h (Fig.2d; 12.68 ± 1.37 in P10 vs. 8.79 ± 0.64h in adults). Moreover, circadian period length was about 1.5h longer in P10 islet cells (25.08 ± 0.16 vs. 23.81 ± 0.22h in adults; Fig.2e). The average amplitude of *Bmal1*-luc oscillations in P10 tended to be lower compared to the adult counterpart but did not reach statistical significance (Fig.2f, 0.19 ± 0.03 vs. 0.27 ± 0.046, p=0.248). However, desynchronization was faster in P10 than in adult islet cells as shown by a decreased circadian amplitude starting from the fifth cycle compared to the second cycle in P10 cells (Fig.2g, p=0.0061). Taken together, these data suggest that while the rhythmic oscillations of *Bmal1*-luc reporter were observed in P10 islet cells synchronized *in vitro*, their properties differed significantly from those of the adult counterpart.

Islet circadian genes are differentially expressed during postnatal maturation.

Since beta-cell postnatal maturation involves drastic changes in the expression of numerous islet genes, mainly involved in stimulus-secretion coupling and cell cycling [1], we determined whether the expression of circadian genes also differs between newborn and adult islets. Real-time PCR measurements revealed that half of the tested genes display different levels in adult compared to immature newborn islets (Fig.3a). The expression of *Clock, Npas2, Bmal1* and *Bmal2* is higher in fully mature islet cells, whereas the levels of *Rev-erbα* and *Per3* are lower. We next examined the kinetics of the changes in the expression of these circadian genes by measuring their levels at different time points during postnatal life. We observed that immediately after birth the expression profiles are stable and are modified after the third postnatal week (Fig.3b-g), i.e. at the time when islet cells become fully functional. Indeed, we previously demonstrated that the nutritional shift occurring at weaning (postnatal day 21) leads to the acquisition of a mature insulin secretory phenotype. This adaptation ensures the release of appropriate amounts of insulin in response to an increased carbohydrate dietary intake [1]. These data reveal that the adult circadian gene expression profile in islets is established once beta-cell maturation has occurred.

miRNAs regulate circadian gene expression.

Nutrient-induced postnatal miRNA modifications promote beta-cell maturation by regulating the expression of metabolic and cell cycle genes [1]. To further determine the contribution of miRNAs to the regulation of islet circadian genes in postnatal life, we used the computational algorithms miRSystem and TargetScan to search for miRNAs targeting the 3′UTR of circadian mRNAs. We included in our computational analysis 17 miRNAs that we have previously investigated for their role in beta-cell maturation [1]. The compiled results revealed that the predicted targets of miR-17-5p and miR-29b-3p are enriched in genes belonging to the circadian machinery. To experimentally verify the computational predictions, we mimicked in islet cells obtained from 10-day-old rats the reduction of miR-17-5p (Supplementary Fig.1a) and the up-regulation of miR-29-3p (Supplementary Fig.1b) occurring during the postnatal maturation of beta-cells. Indeed, the decrease of miR-17-5p resulted in an increase of *Clock* and *Npas2* mRNA levels (Fig.4a, b). Dual luciferase reporter assays in the rat beta-cell line INS832/13 confirmed that miR-17-5p directly targets the 3′UTR of *Clock*, but not *Npas2* (Fig.4c; supplementary Fig.1c). Thus, the effect of miR-17-5p

on *Npas2* expression is most likely indirect. As predicted by the computational programs, the increase of miR-29b-3p in newborn islet cells resulted in a reduction in *Per3* expression (Fig.4d) and suppressed the luciferase activity produced from the *Per3*-3'UTR construct (Fig.4e; supplementary Fig.1d). These findings confirm that miR-29b-3p directly inhibits the expression of *Per3*, the antagonistic regulator of the CLOCK/BMAL1 heterodimer complex. We then assessed whether the expression of miR-17-5p and miR-29b-3p follows an oscillatory pattern across 24h. As expected, the levels of miR-17-5p were lower and those of miR-29b-3p higher at all time points in adult islets [1]. However, none of the miRNAs displayed circadian oscillatory patterns (Fig.4f,g).

Beside miR-17-5p and miR-29b-3p, several other miRNAs displaying expression changes during postnatal beta-cell maturation are predicted to target at least one core clock gene. To obtain a comprehensive picture of the potential impact of miRNAs on the expression of core clock components, we used a computational approach to search for all the predicted interactions between the 62 miRNAs displaying expression changes during the functional maturation of beta-cells [1] and the 12 clock genes investigated in this study (Fig.1). MiRNAs and core clock genes were found to be connected by a complex regulatory network (Fig.5) with more than 40 miRNAs showing anti-correlated expression changes with their respective targets. As expected, most miRNAs were predicted to regulate the level of multiple clock genes and several clock genes were found to be targeted by multiple miRNAs. Of note, 20 different miRNAs displaying reduced expression changes upon weaning were found to potentially contribute to the observed rise in the level of *Clock* in mature beta-cells (Fig.5). These findings indicate that the islet circadian gene expression profile is probably not determined by a single miRNA but results from the cooperative action of multiple miRNAs involved in the functional maturation of beta-cells.

Discussion

Emerging evidence indicates that the cell-autonomous circadian clocks operating in the endocrine pancreas play an essential role in coordinating insulin secretion and islet cell transcriptome in a cyclic-dependent manner [5, 6, 33-36]. However, so far the rhythmicity and expression profiles of clock genes in pancreatic islets and the mechanisms regulating them throughout postnatal beta-cell maturation have not been investigated. The present study highlight drastic differences in the expression and temporal profiles of the core-clock genes between 10-day-old and 3-month-old rat islets. Importantly, we found that miR-17-5p and miR-29b-3p directly regulate the core circadian genes *Clock* and *Per3* throughout postnatal islet maturation, and are likely to be involved in establishing functional autonomous molecular clock. Our data suggest that the regulatory network connecting miRNAs and clock genes is most probably far more complex and the establishment of a fully operational circadian clock is unlikely to be explained by single miRNA-target associations. Indeed, several other miRNAs involved in the functional maturation of beta-cells are predicted to regulate at least one of the core-clock genes and display anti-correlated expression changes with their putative targets. Additional studies will be needed to experimentally verify these computational predictions and to precisely delineate the interconnection between miRNAs and the circadian clock in the context of postnatal beta-cell maturation.

We demonstrated that circadian expression patterns of clock components are not fully established in immature newborn islets. Of note, while *in vivo* circadian oscillations of newborn islet cells were strongly dampened, oscillations of the circadian reporter could still be induced *in vitro* in P10 islet cells by forskolin synchronization (compare Fig.1 to Fig.2). These findings suggest that the lack of oscillations observed in newborns is a consequence of the desynchronization of the cells rather than an intrinsic incapacity of clock genes to undergo circadian expression changes. However, the oscillatory profiles observed *in vitro* have distinct characteristics in P10 cells compared to the adult counterpart. These changes comprised the phase advance of the first circadian peak (Fig.2c), which may suggest the altered immediate resetting response. The differences between P10 and adult islet cell clocks were not limited to the kinetics of the first circadian cycle, but were extended to the circadian cycle characteristics such as phase, amplitude, and period length (Fig.2d-f), including faster decrease in the amplitude in the P10 oscillations, which may suggest more rapid desynchronization for these cells following the initial pulse. In agreement with our observations, the reported phase shift of the circadian oscillator Per1 in rat liver, thyroid, and

pineal gland throughout development [19] may highlight a ubiquitous delay of the clock gene oscillations in the maturating peripheral tissues after birth. Despite the circadian clock machinery is already expressed at a cellular level at very early developmental stages - although initially only at low basal levels - it was proposed that circadian rhythm ontogenesis is strongly influenced by changes in the environmental conditions and in organ function occurring during development [19, 37, 38]. It is well established that both mature beta and alpha-cells display cell-autonomous circadian rhythms of core-clock and functional metabolic genes [6, 7, 39]. Consistently, we observed that forskolin-induced oscillations of clock genes are elicited *ex vivo* despite the absence of *in vivo* circadian rhythms in newborn islets. These data were obtained in whole islets. Hence, these changes may occur and have an effect on one or more islet cell types. Nevertheless, beta-cells constitute the main cell type in both P10 and adult islets, suggesting that modifications in the circadian gene oscillation profiles and overall mRNA levels contribute to beta-cell maturation. To further elucidate the mechanism of beta-cell clock maturation, *in vitro* experiments implying the single cell analysis of the P10 and adult islet circadian bioluminescence profiles will be required. Taken together, our results suggest that the initiation of islet circadian rhythms might rely on environmental factors, comprising blood-born signals. Although further investigations are needed to determine the potential involvement of hormonal factors and neural inputs in the establishment of circadian rhythms, our study reveals that miRNAs are part of the mechanisms contributing to the post-transcriptional control of clock genes in immature islets. We have shown previously that miRNA modifications are driven by the shift in food supply occurring at weaning when the high-fat maternal milk is replaced by a carbohydrate-enriched diet. In view of this, it is tempting to speculate that the nutritional switch is not only responsible for islet miRNA changes and the subsequent functional beta-cell maturation but also for adaptive circadian clock ontogenesis. Indeed, the attenuated clock gene expression and phase in the islets during early postnatal life suggest that the circadian regulation of insulin secretion is not yet necessary for maintaining blood glucose homeostasis since newborn animals fed a low carbohydrate-diet are normoglycemic. Accordingly, disruption of circadian rhythmicity of clock genes induces beta-cell dysfunction, impairment in glucose metabolism and the release of inappropriate amounts of insulin [33, 36]. Before weaning, the low glucose responsiveness of newborns is thought to be the consequence of reduced glucose oxidation and metabolite production leading to an imbalanced NADPH/NADP [40]. NADPH and the subsequent activation of essential transcription factors is a strong regulator of clock gene activation and has the potential to phase-shift circadian rhythms [41, 42]. This supports our hypothesis that

circadian clock ontogenesis is rather needed once beta-cells have to face the rise in insulin needs required to match the increased carbohydrate supply and hence, islet clock is required to coordinate beta-cell function with circadian energy homeostasis. Our study also unveils that the expression of core clock genes in postnatal rat islets reaches similar levels to those of adults within 30 days of age. The differential rhythmicity and expression of clock genes observed in immature compared to adult islets have been suggested to apply also to other metabolic organs in early life. Indeed, previous studies reported a gradual development of the circadian clock during the postnatal period with temporal differences in the appearance of clock amplitude oscillations between the central nervous system and the heart in mice [43]. This raises the hypothesis that the time-frame of islet clock establishment might be cell type-specific and directly related to the capacity of the beta-cells to release insulin in response to nutrients and, in particular, to glucose, permitting to meet the increased insulin needs caused by food transition.

The islet miRNA changes taking place during postnatal beta-cell maturation - that we detailed in a previous study [1] - are unlikely to be dictated by the central clock pacemaker in the suprachiasmatic nucleus, which is itself activated by light exposure [44]. In contrast, miRNA modifications occur between postnatal days P20 and P31 when the transition from the fat-enriched maternal milk to a high-carbohydrate diet takes place. In line with this, Sladek and colleagues showed that the amplitude in the oscillations of clock genes in rat liver are not yet established at the age of 20 days and achieve a mature state only around P30 [24]. Similarly, circadian expression of intestinal disaccharidase enzymes achieves the levels observed in adult rats only several days after weaning [45]. Previous findings suggest that the instauration of clock gene expression and rhythmicity in peripheral tissues such as the endocrine pancreas is entrained by the periodicity of meal, which is acquired during adulthood, rather than to light [46]. Indeed, photo-entrainment is already functional at birth [47], whereas rhythmicity of timely regulated food intake seems to be established after the shift to solid food, re-enforcing the necessity of getting a regulated insulin release in a circadian manner after the transition of nutritional supplies occurring at weaning. Food-anticipatory activities are defined as food-seeking behaviors in anticipation of mealtime, including physiological and hormonal activations in response to daily temporal windows of feeding time [48, 49]. Notably, global and brain-specific deletions of the clock component *Rev-erbα* were reported to impair the 24-hour pattern of food entrainment in mice [50]. We could speculate that in newborns the lack of regulated temporal pattern of food intake justify

the absence of a circadian control of the secretory activity of beta-cells. This raises the possibility that circadian clock entrainment and feeding time dictate the already described phasic insulin release from adult islets [7].

In conclusion, we have unveiled the contribution of miRNAs to the circadian clock regulation in pancreatic islets during the postnatal period. The suggested plethora of known and yet-to-be-discovered signaling pathways linking the chronobiology of insulin secretion with miRNAs, points out that we have just begun to scratch the surface of understanding the intercross between the circadian clock and postnatal beta-cell maturation.

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Disclosure Summary: The authors have nothing to disclose.

Contribution statement

CJ and ART generated and analyzed the data, wrote the manuscript and approved its final version. CG, JS, SG, VP and CS contributed to the generation and the analysis of the data, critically revised the manuscript and approved its final version. CD contributed to the analysis and the interpretation of the data, critically reviewed the manuscript and approved its final version. RR designed the experiments, interpreted the data, wrote the manuscript and approved its final version. RR is the guarantor of this work.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Figure legends

Figure 1. Rhythmic expression profile of genes involved in circadian clock in newborn and adult islets. The islets of 10-day old (closed circles) and adult (open circles) rats were isolated and the RNA extracted at 4 h intervals across the 12:12 LD cycle. Transcript expression was determined by RT-qPCR. Fold-change values are displayed as relative abundance to that of 18S amplified within the same sample. 2-way ANOVA was used to determine variance with respect to time and group (mean ± SD, n=4 rats per group per time point, *P<0.05 between groups).

Figure 2. Newborn and adult islet cells synchronized *in vitro* exhibit distinct circadian oscillation profiles. Pancreatic islets were isolated from 10-day-old (P10) and 3-month-old adult rats. Islet cells were transduced with lentiviral particles expressing Bmal1-luciferase (Bmal1-luc), synchronized with forskolin pulse, and transferred to the Lumicycle for bioluminescence recording. (**a**) Raw data profiles (counts/min) from a parallel recording of islet cells derived from P10 and adult animals. (**b**) Average detrended bioluminescence profiles for n=3 independent experiments (1 pooled litter of 10-12 pups per experiment) for P10 islet cells, and n=3 independent experiments (1 animal per experiment) for adult islet cells. (**c**) Zenith (peak) time for the first peak of Bmal1-luc detrended bioluminescence (**b**) following forskolin synchronization; (**d**) Average circadian phase of three consecutive peaks starting from the second peak and normalized to 24h day period; (**e**) Circadian period length; (**f**) Circadian amplitude, averaged for all the cycles starting from the 2^{nd} cycle (left graph), or presented individually for each cycle (right graph; black bars represent P10 islet cells, and white bars express adult cells). Data in **c-f** are expressed as mean ± SEM, n=3 independent experiments (1 pooled litter of 10-12 pups per experiment) for P10 islet cells and n=3 independent experiments (1 animal per experiment) for adult islet cells. p<0.05 unpaired, two-tailed Student's *t*-test. Panel f right graph: one-way ANOVA test was applied to P10 and adult graphs separately, indicating significant (p=0.0061) decrease of amplitude in P10 islet cells, but not in adults (p=0.5390).

Figure 3. Modifications of islet clock gene expression throughout postnatal life. (**a**) qRT-PCR were performed using islet RNAs of 10-day-old versus 3 month-old rats isolated between 8:00 and 10:00. The expression of the indicated genes in adult islets is presented as the ratio to the level measured in newborn islets (dotted line) and represent mean ± SD of 4 rats per group. *P<0.05 vs. newborn rats, ANOVA. (**b, c, d, e, f, g**) qRT-PCR were performed using samples of rats from the indicated ages. Data are means ± SD (n=4 per group). Statistical difference

from 10-day-old rats was assessed by one-way ANOVA with a Dunnett post-hoc test: *P<0.05. Npas2 expression: p=0.06 between P10 and adult islets.

Figure 4. miR-17-5p and miR-29b-3p regulation of clock genes and oscillation profiles. (**a, b, d**) Dispersed 10-day-old rat islet cells were transfected with anti-miRNAs to inhibit (**a, b**) or with oligonucleotide mimics (**d**) to overexpress the miRNAs. mRNA levels assessed by qRT-PCR are shown as fold changes (n=4 to 6). Data are means ± SD. *p<0.05, Student's *t*-test. (**c, e**) Direct interaction of miR-17-5p (**c**) and miR-29b-3p (**e**) with their putative targets was assessed by luciferase reporter assays in INS832/13 cells. Vectors containing the 3'UTR of *Clock*, *Npas2*, and *Per3* were co-transfected with either a control oligonucleotide or oligonucleotides mimicking the miRNAs of interest. An empty vector was used as control. Data are means ± SD (n=4) and statistical significance was calculated by ANOVA. (**f, g**) The level of miR-17-5p and miR-29b-3p in 10-day-old (closed circles) and adult (open circles) rat islets isolated at 4h intervals across the 12:12 LD cycle was determined by qRT-PCR. The data (mean ± SD, n=4 per group per time point, *p<0.05 between groups) are displayed as relative abundance to that of U6 amplified within the same sample.

Figure 5. Core-clock mRNA-miRNA network during beta-cell maturation. The potential interactions between the miRNAs displaying expression changes during postnatal beta-cell maturation and circadian clock genes were computationally predicted as described in the experimental procedures. Green and red edge symbols indicate up- and down-regulation between adult and newborn rat islets, respectively. Opposite expression changes between the miRNAs and their putative targets suggestive of functional interactions are depicted with bold blue lines while changes in the same direction are shown with pink lines. Clock genes are shown with square symbols while miRNAs are presented as diamond symbols.

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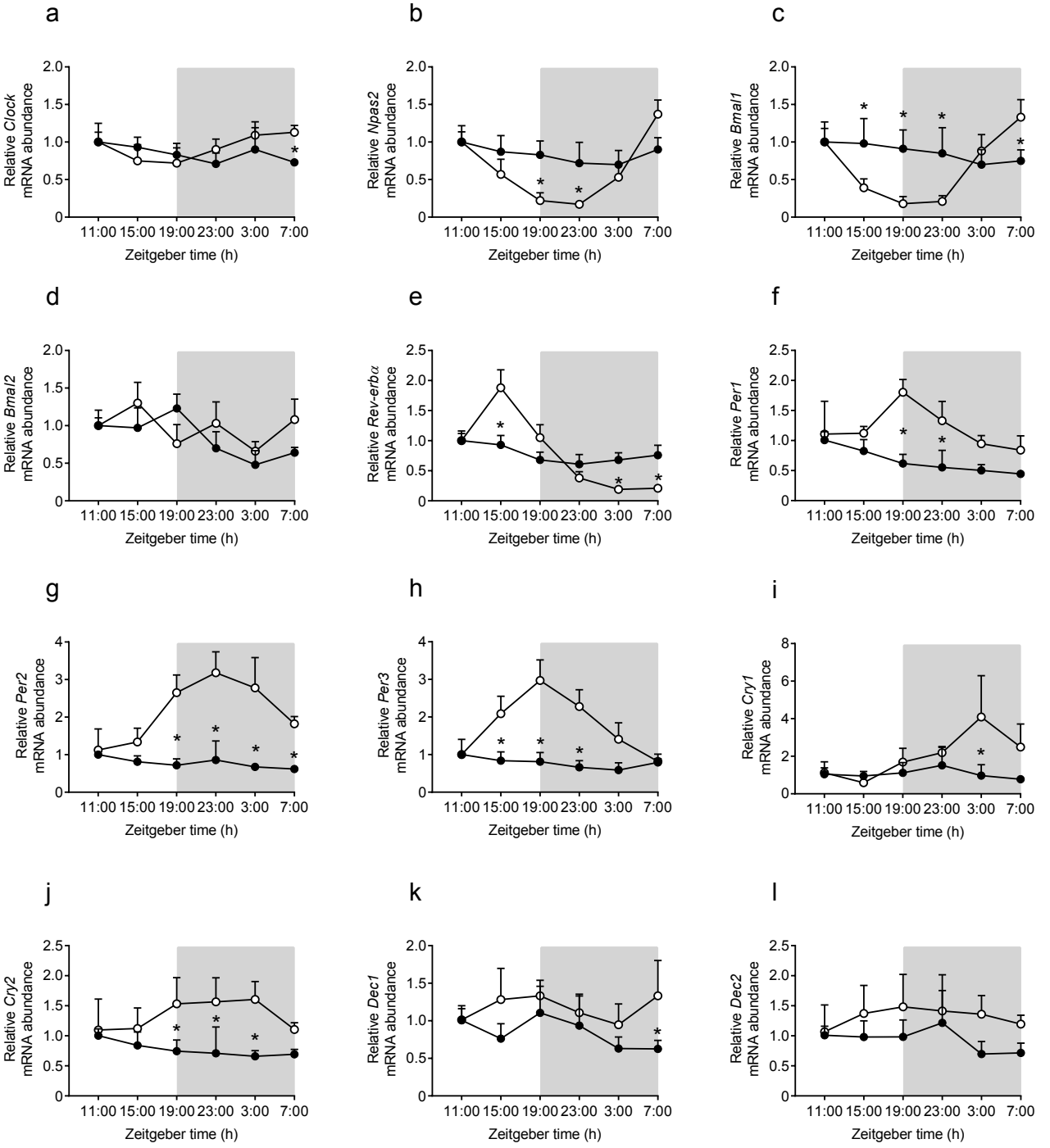
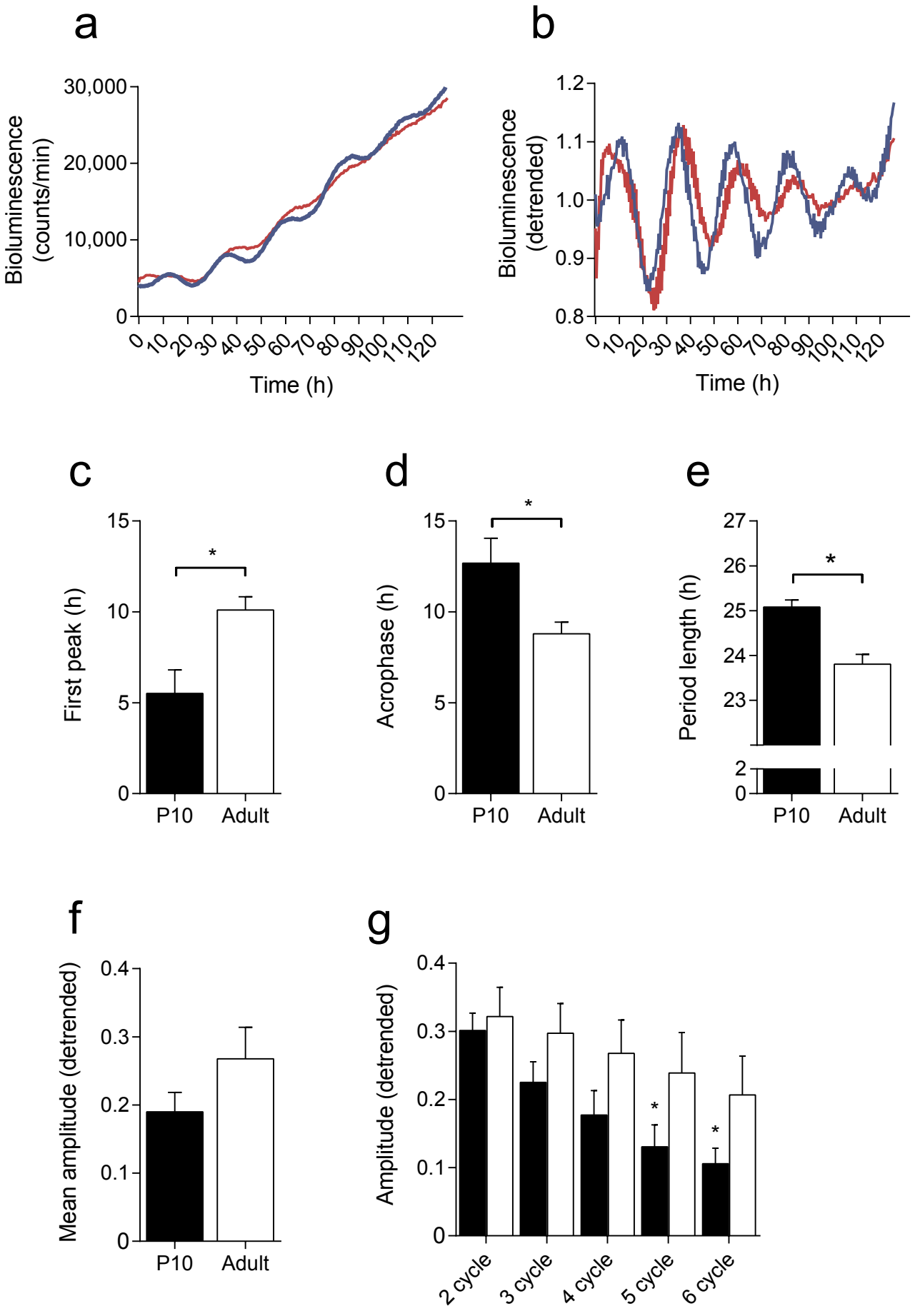

a
b
c
d
e
f
g
h
i
j
k
l
Relative Clock mRNA abundance
Relative Npas2 mRNA abundance
Relative Bmal1 mRNA abundance
Relative Bmal2 mRNA abundance
Relative Rev-erbα mRNA abundance
Relative Per1 mRNA abundance
Relative Per2 mRNA abundance
Relative Per3 mRNA abundance
Relative Cry1 mRNA abundance
Relative Cry2 mRNA abundance
Relative Dec1 mRNA abundance
Relative Dec2 mRNA abundance
11:00 15:00 19:00 23:00 3:00 7:00
Zeitgeber time (h)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20,000
10,000
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Time (h)
b
Bioluminescence (detrended)
1.2
1.1
1.0
0.9
0.8
0 10 20 30 40 50 60 70 80 90 100 110 120
Time (h)
c
First peak (h)
15
10
5
0
*
P10
Adult
d
Acrophase (h)
15
10
5
0
*
P10
Adult
e
Period length (h)
27
26
25
24
23
2
0
*
P10
Adult
f
Mean amplitude (detrended)
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0.3
0.2
0.1
0
P10
Adult
g
Amplitude (detrended)
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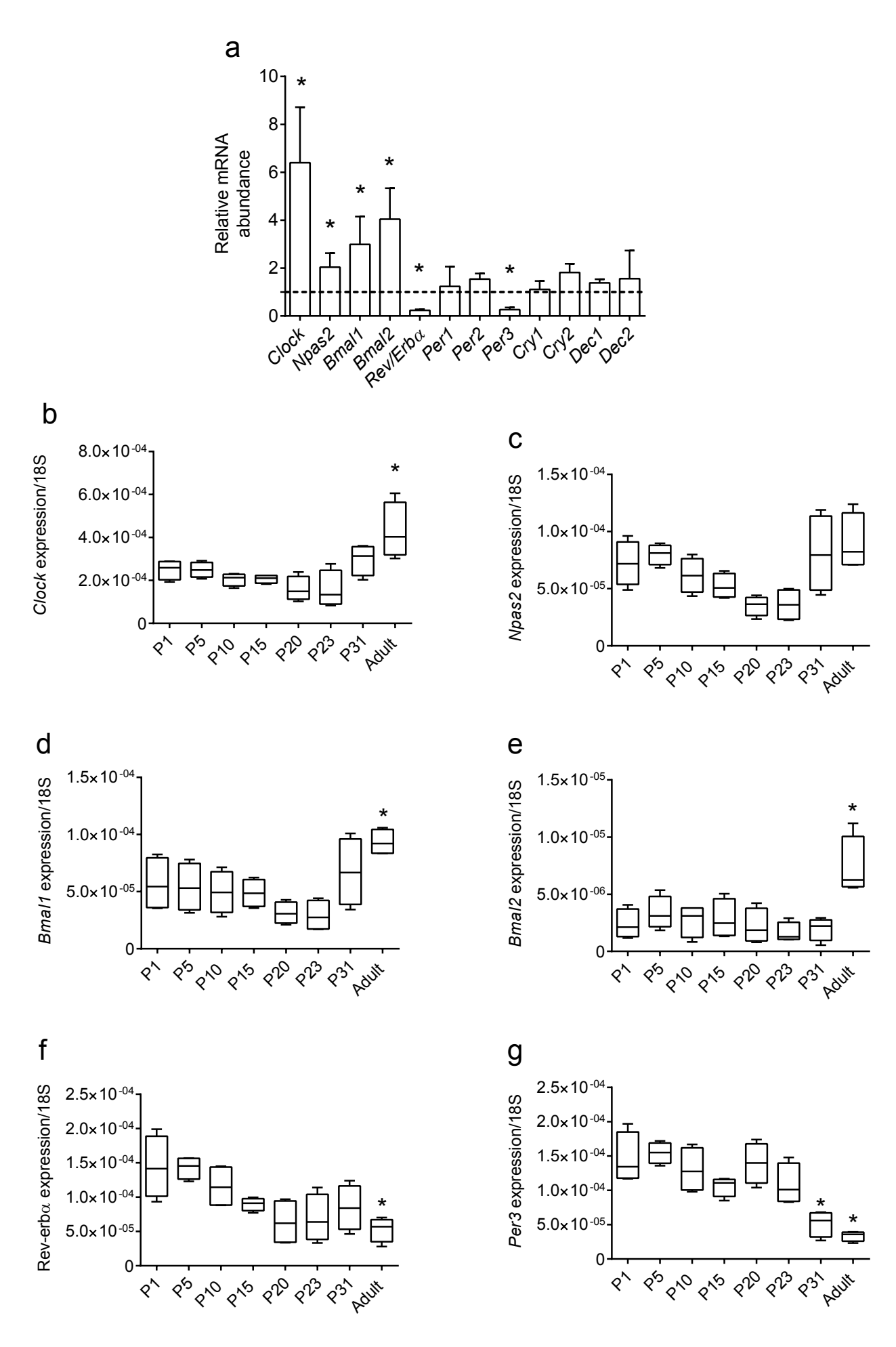
0.3
0.2
0.1
0
*
*
2 cycle
3 cycle
4 cycle
5 cycle
6 cycle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Relative mRNA abundance
10
8
6
4
2
0
*
Clock
Npas2
Bmal1
Bmal2
Rev/Erbα
Per1
Per2
Per3
Cry1
Cry2
Dec1
Dec2
b
Clock expression/18S
8.0x10-04
6.0x10-04
4.0x10-04
2.0x10-04
0
P1
P5
P10
P15
P20
P23
P31
Adult
c
Npas2 expression/18S
1.5x10-04
1.0x10-04
5.0x10-05
d
Bmal1 expression/18S
1.5x10-04
1.0x10-04
5.0x10-05
e
Bmal2 expression/18S
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1.0x10-05
5.0x10-06
f
Rev-erbα expression/18S
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2.0x10-04
1.5x10-04
1.0x10-04
5.0x10-05
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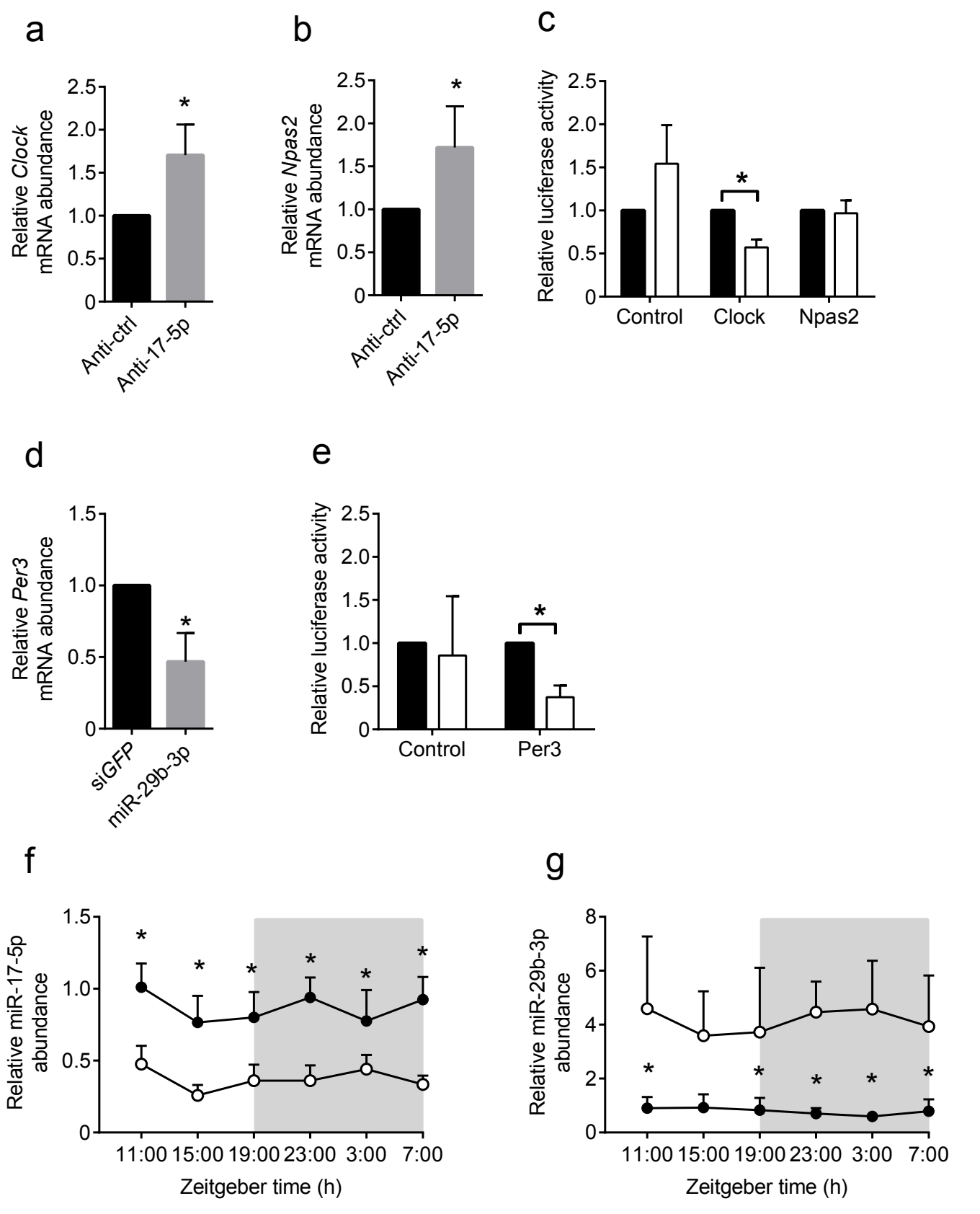
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Relative Clock mRNA abundance
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1.5
1.0
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0
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Anti-ctrl
Anti-17-5p
b
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2.0
1.5
1.0
0.5
0
*
Anti-ctrl
Anti-17-5p
c
Relative luciferase activity
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2.0
1.5
1.0
0.5
0
*
Control
Clock
Npas2
d
Relative Per3 mRNA abundance
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1.0
0.5
0
*
siGFP
miR-29b-3p
e
Relative luciferase activity
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2.0
1.5
1.0
0.5
0
*
Control
Per3
f
Relative miR-17-5p abundance
1.5
1.0
0.5
0
*
11:00
15:00
19:00
23:00
3:00
7:00
Zeitgeber time (h)
g
Relative miR-29b-3p abundance
8
6
4
2
0
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11:00
15:00
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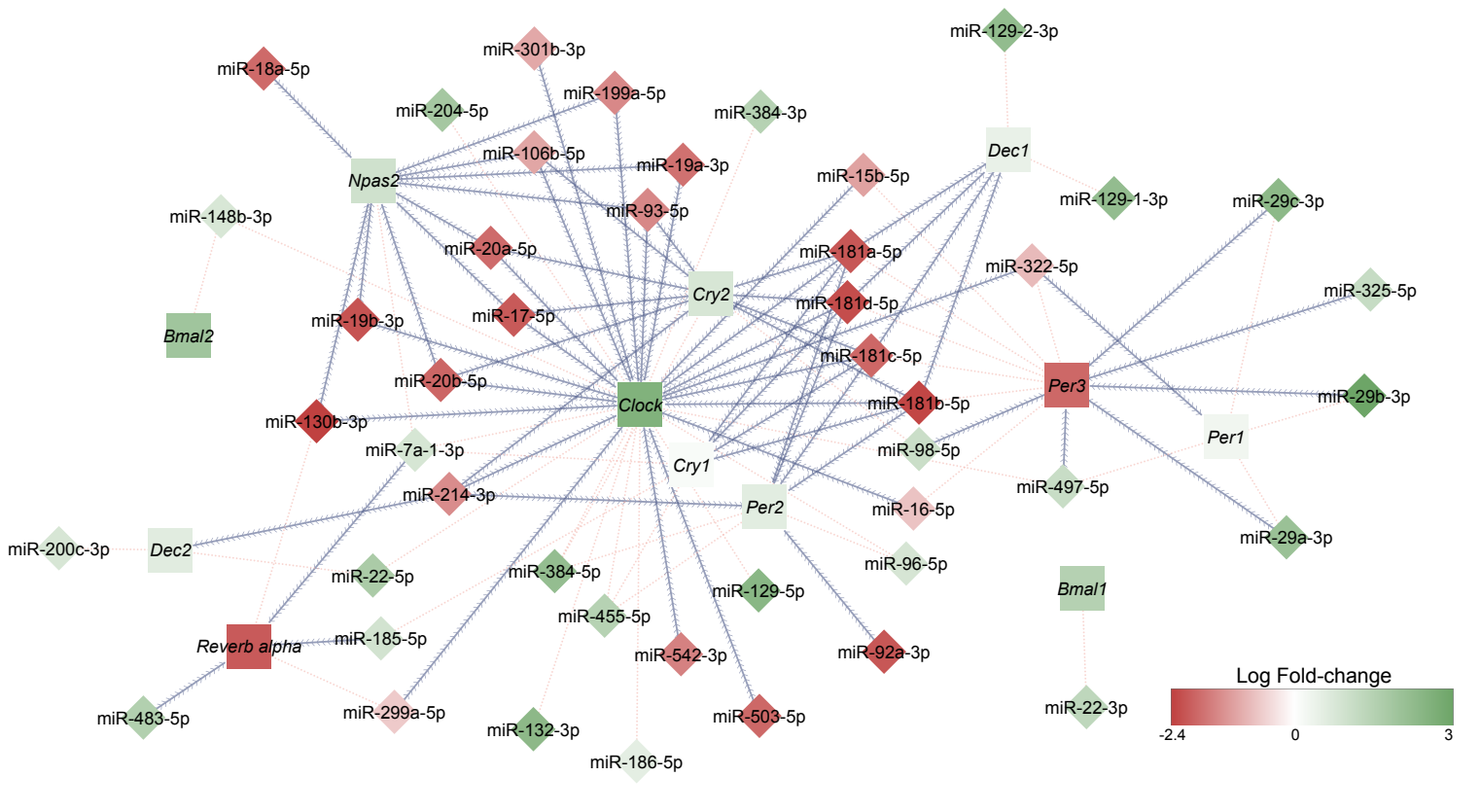
23:00
3:00
7:00
Zeitgeber time (h)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miR-18a-5p
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miR-384-3p
Dec1
miR-106b-5p
miR-19a-3p
Npas2
miR-15b-5p
miR-129-1-3p
miR-29c-3p
miR-148b-3p
miR-93-5p
miR-20a-5p
miR-181a-5p
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Cry2
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Bmal2
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Per1
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Cry1
miR-497-5p
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Per2
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Dec2
miR-96-5p
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Bmal1
miR-129-5p
miR-455-5p
Reverb alpha
miR-185-5p
miR-542-3p
miR-92a-3p
miR-483-5p
miR-299a-5p
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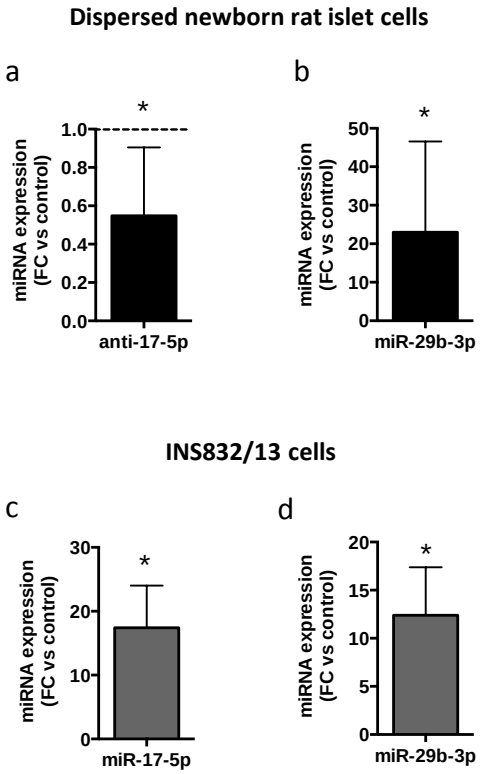
miR-22-3p
miR-132-3p
miR-186-5p
Log Fold-change
-2.4
0
3

Supplementary figure 1. Down- and up-regulation of microRNAs. (a, b) 10-day-old rat islets were dissociated and transfected for 48h. To reduce miR-17-5p we used single-stranded anti-miRNAs or a scrambled anti-miR sequence as control. To increase the level of miR-17-5p and miR-29b-3p either in P10 islet cells (**b**) or in INS832/13 cells (**c, d**), we used oligonucleotide mimics or an siRNA duplex against GFP as control. Results are expressed as fold changes (FC) *versus* the control anti-miR (n=3 to 5). Statistical differences were determined by Student's t-test (*P<0.05).



Supplementary Table 1. Primer sequences.

Genes	Forward primer	Reverse primer	Species
18S	5’-GGAATTATTCCCCATGAACG-3’	5’-GGCCTCACTAAACCATCCAA-3’	R, M, H
Bmal1	5’-GCACTCACACATGGTTCCAC-3’	5’-CATTCCGCAAGGTGTCCTAT-3’	R
Bmal2	5’-AGCCCACAAACTGGACAAAC-3’	5’-TCCTTCACATCCAACCACAA-3’	R
Clock	5’-GAGAACTTGGCGTTGAGGAG-3’	5’-GGAAGGGTCTGAGACTGCTG-3’	R
Cry1	5’-TCAGTTGGGAAGAAGGGATG-3’	5’-TTTTGCAGGGAAGCCTCTTA -3’	R
Cry2	5’-AGACAGGCTTCCCTTGGATT-3’	5’-AGGGCAGTAGCAGTGGAAGA -3’	R, M, H
Dec1	5’-CTCCTGCAGGGTAGTGCTTC-3’	5’-CGCTCTTGAAGTAGGGTTGC-3’	R
Dec2	5’-AAGGCAAAAACCGAGACCTT -3’	5’-AGTAGCAACAGCAGCAGCAA -3’	R
Npas2	5’-GGAGTCCAGAAGCAAACAGC-3’	5’-GTTCTTTCCCCATTCTGCAA-3’	R
Per1	5’-TTCCCTGTTTTGTCCTCCAC-3’	5’-TGCTGTTTGCATCAGTGTCA-3’	R
Per2	5’-AGGTACCTGGAGAGCTGCAA-3’	5’-GGTGAGGGACACCACACTCT-3’	R
Per3	5’-GAAGCACAAACGGAAGAAGC-3’	5’-GGAGTCCCCTACTCCCTGAG-3’	R
Rev/Erb alpha	5’-CTGGAGGGCTGCAGTATAGC-3’	5’-AGGGTCGTCATGTCTTCACC-3’	R

Rat (R); Mouse (M); Human (H)

Mature miRNA sequences.

Name	Mature miRNA sequences
rno-miR-17-5p	5’-CAAAGUGCUUACAGUGCAGGUAG-3’
rno-miR-29b-3p	5-’UAGCACCAUUUGAAAUCAGUGUU-3’