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Post-transcriptional modulators and mediators of the circadian clock

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Abstract

The endogenous circadian timekeeping system drives ~24 hour rhythm in the gene expression and rhythmically coordinates the physiology, metabolism and behavior in a wide range of organisms. Regulation at various levels is important for the accurate functioning of this circadian timing system. The core circadian oscillator comprises of an interlocked transcriptional-translational negative feedback loop (TTFL) that impose a substantial delay between the accumulation of clock gene mRNA and its protein to generate 24-hour oscillations. This TTFL mediated daily oscillation of clock proteins are further fine-tuned by post-translational modifications that regulate the clock protein stability, interaction with other proteins and subcellular localization. Emerging evidence from various studies indicates that besides TTFL and post-translational modifications, post-transcriptional regulation plays a key role in shaping the rhythmicity of mRNAs and to delay the accumulation of clock proteins in relation to their mRNA. In this review, we will summarize the current knowledge on the importance of post-transcriptional regulatory mechanisms such as splicing, polyadenylation, the role of RNA binding proteins, RNA methylation and microRNAs in the context of shaping the circadian rhythmicity in *Drosophila* and mammals. In particular, we will discuss microRNAs, an important player in post-transcriptional regulation of core-clock machinery, circadian neural circuit, clock input and output pathways. Furthermore we will provide an overview of the microRNAs that exhibit diurnal rhythm in expression and their role in mediating the rhythmic physiological processes.

Keywords

circadian; microRNA; post-transcriptional; input; output; mRNA

Introduction

Living organisms evolved by adapting to the daily cyclic fluctuations of environmental cues imposed by the rotation of the earth on its own axis. During the course of evolution, almost all organisms attained an endogenous timekeeping system to anticipate and adapt to these fluctuating environmental conditions (Johnson et al. 2003). This endogenous circadian

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clock perceives the environmental signals such as light, temperature and food through the input pathways. These input signals are transmitted to the central pacemaker that generates self-sustained oscillations of ~24 hours (h) and this timing information is further relayed to various downstream output pathways to generate overt rhythms in diverse behavioral, biochemical and physiological processes (Takahashi et al. 2001; Quintero et al. 2003; Lowrey and Takahashi 2004; Dubruille and Emery 2008; Allada and Chung 2010; Lowrey and Takahashi 2011; Mehta and Cheng 2013). The circadian pacemaker in *Drosophila* and mammals mainly comprises of conserved interlocked transcriptional/ translational feedback loops (TTFL) that drives self-sustained ~24-hour oscillation in the gene expression of the core clock components (Hardin et al. 1990; Allada et al. 2001; Yu and Hardin 2006; Lowrey and Takahashi 2011).

In *Drosophila melanogaster*, the TTFL consists of the positive limb with CLOCK (CLK) and CYCLE (CYC) heterodimeric transcription factor complex that activates the transcription of the repressor complex PERIOD (PER) and TIMELESS (TIM) (Figure 1). This repressor complex PER-TIM subsequently closes the loop by inhibiting the transcriptional activity of the positive limb components CLK-CYC (Konopka and Benzer 1971; Sehgal et al. 1994; Darlington et al. 1998; Allada et al. 1998; Rutila et al. 1998; Benito et al. 2007; Hardin 2011; Hurley et al. 2016). Additionally, the CLK-CYC complex activates the transcription of secondary feedback loop components *vrille* (*vri*), *Pdp1ε* and *clockwork orange* (*cwo*). VRI and PDP1ε rhythmically inhibit and activate the transcription of *Clk* respectively whereas CWO inhibits CLK-CYC transcriptional activity (Glossop et al. 2003; Cyran et al. 2003; Kadener et al. 2007). In addition to these primary and secondary feedback loops, CRYPTOCHROME (CRY) governs the light-mediated degradation of TIM and triggers the resetting of the circadian clock (Stanewsky et al. 1998; Dolezelova et al. 2007). In mammals, the transcriptional activators CLOCK and BMAL1 form the positive limb to activate the transcription of the PER (PER1, PER2, PER3) and CRY (CRY1, CRY2) repressor complex (King et al. 1997; Gekakis et al. 1998; Bunger et al. 2000; Zheng et al. 2001) (Figure 2). This repressor complex in turn inhibits the activity of CLOCK-BMAL1 (Kume et al. 1999; Griffin 1999; Sato et al. 2006). Rhythmic expression of BMAL1 is additionally regulated by a secondary feedback loop consisting of REV-ERBα and RORα proteins that inhibit or activate *Bmal1* transcription respectively (Preitner et al. 2002; Guillaumond et al. 2005).

Apart from the TTFL mediated clock gene expression regulation, evidence from several studies indicates the importance of different post-translational modifications such as phosphorylation, acetylation, SUMOylation, ubiquitination, O-GlcNAcylation and poly (ADP-ribosyl)ation in mediating the stability, subcellular compartmentalisation and the rhythmic abundance of clock proteins for establishing the pace of the circadian clock with ~24h rhythmicity (Martinek et al. 2001; Lee et al. 2001; Miyazaki et al. 2004; Cyran et al. 2005; Hirayama et al. 2007; Asher et al. 2010; Reischl and Kramer 2011; Weber et al. 2011). For instance, kinase mediated phosphorylation governs the stability of clock proteins in both flies and mammals (Reischl and Kramer 2011). In *Drosophila,* DOUBLE TIME (DBT) and SHAGGY phosphorylate PER and TIM respectively to fine-tune circadian oscillations (Kloss et al. 1998; Martinek et al. 2001; Cyran et al. 2005). Another possible mode of circadian clock regulation is at the post-transcriptional level. In addition to understanding the importance of post-transcriptional regulation in the robustness of the

circadian timing system, it is important to understand the role of different RNA specific regulatory mechanisms in governing the time lag between clock gene mRNA and protein oscillation. Recent studies recognised the relevance of a new class of post-transcriptional regulators, microRNAs (miRNAs) in regulating the diverse aspects of circadian rhythms (Kojima et al. 2011; Mehta and Cheng 2013; Mendoza-Viveros et al. 2017; Xue and Zhang 2018). In this review, we will provide an overview of the current knowledge on the post-transcriptional regulation of the circadian clock by emphasizing the findings on circadian relevant microRNAs in *Drosophila* and mammalian model systems. We will further discuss about the miRNAs that undergo circadian cycling and its impact on rhythmic physiological processes.

Post-transcriptional regulation of circadian rhythm

Multiple layers of post-transcriptional regulation ranging from splicing, polyadenylation at the 3'end and transport of mRNA from the nucleus have been reported to affect the phase, amplitude and free running periodicity of circadian rhythm (Green 2018). 3' untranslated regions (3' UTRs) of mRNAs have specific binding sites for RNA binding proteins (RBPs) or microRNAs (miRNAs) and these complicated series of post-transcriptional mechanisms collectively plays a key role in shaping the mRNA expression rhythmicity and in delaying the accumulation of clock proteins in relation to their respective mRNAs (Chen et al. 2013). This section summarizes various post-transcriptional regulatory processes and their relevance in the context of circadian rhythmicity.

Post-transcriptional regulation of circadian clock by alternative splicing

The functional importance of alternative splicing on the circadian clock has been recognized in *Drosophila* by studying the alternative splice variant isoforms of core clock genes. Studies showed that light and temperature modulate the alternative splicing of an intron (dmpi8 intron) located in the 3' UTR of *period* mRNA and thus conferring the fly the ability to redistribute its activity in accordance with the seasonal changes in temperature and day length (Majercak et al. 1999; Majercak et al. 2004). Lower temperature enhances the splicing of dmpi8 intron. This enhanced splicing is associated with an advance in the steady-state phase of the PER protein that attributes to the earlier evening activity of flies under cold temperature (Majercak et al. 1999). Further studies showed that in addition to low temperature, short photoperiod also enhances dmpi8 intron splicing and *no receptor potential A (norpA)* gene that encodes phospoholipase C plays a non-photic role in regulating the dmpi8 splicing (Collins et al. 2004; Majercak et al. 2004). In addition, the serine/arginine family of protein splicing factor B52/SRp55 enhances the dmpi8 splicing efficiency and regulates mid-day sleep in *Drosophila* (Zhang et al. 2018).

Apart from affecting the *per* mRNA alternative splicing, temperature alters the splicing pattern of the core clock component *timeless* in *Drosophila*. Alternative splicing generates four major isoforms of *tim* (Martin Anduaga et al. 2019). The levels of the canonical transcript *tim-long* was reduced under lower temperature (18˚C) whereas the production of two *tim* cold-specific isoforms such as *tim-cold* and *tim-short and cold* were up-regulated under lower temperature. At higher temperature (29°C) *tim-medium* was abundantly present.

The alternative splicing of *tim* intron sequences regulates the level of TIM at various temperatures and acts as the thermal sensor to adapt to the temperature changes by regulating the daily pattern of locomotor activity (Martin Anduaga et al. 2019). Further studies revealed that the splicing factor P-element somatic inhibitor (PSI) regulates the alternative splicing of *tim* and governs the pace of the circadian clock (Foley et al. 2019). Taken together, these studies collectively elucidated the importance of alternative splicing of *per* and *tim* mRNA on the environmental adaptation of the circadian timekeeping system.

In mice, the first evidence for light-induced rhythmic alternative splicing associated with the circadian clock was reported for the mRNA of U2-auxiliary-factor 26 (U2AF26). A circadian light-inducible splicing switch excludes exons 6 and 7 inducing a frameshift in *U2af26* resulting in translation past into the 3'UTR and generates a C terminus with the TIM homology domain. This alternative U2AF26 isoform controls the stability of PER1 protein and clock resetting (Preußner et al. 2014). Additionally, temporal analysis of SCN by using RNA sequencing characterized a novel isoform of clock gene *Cry1* expressed antiphasic to the canonical *Cry1* oscillation that may be important in the SCN function (Pembroke et al. 2015). Studies in the human keratinocyte cell line provided the first evidence for the presence of the *Per2S* splice variant of the human *Per2* gene (Avitabile et al. 2014). Although studies in mammalian system suggest that alternative splicing plays a key role in stabilizing the clock against the light changes (Preußner et al. 2014), the physiological importance of splicing event of various clock pre-mRNAs including *Cry1* and *Per2* remains elusive.

Relevance of poly(A) tail length in circadian rhythmicity

Among the post-transcriptional regulations, the poly(A) tail 3' modification is not only important in mediating the mRNA stability and translation efficiency but also impacts the rhythmic gene expression in the context of circadian rhythmicity (Grima et al. 2019). Recent evidence suggests that mRNA with shorter poly(A) tails are translated more efficiently than the mRNA molecules with longer tails (Lima et al. 2017). In *Drosophila*, CCR4-NOT complex deadenylates mRNA and regulates the poly(A) length (Temme et al. 2014). Studies showed that the deadenylase component of CCR4-NOT complex encoded by *Pop2* (homolog of *Schizosaccharomyces pombe caf1*) govern the mRNA oscillation and protein levels of the core clock gene *timeless* by regulating the polyadenylation of its mRNA. POP2 shortens the *tim* mRNA poly(A) tail and thus modulates the *tim* mRNA and protein level. Thus POP2 dependent deadenylation mechanism strongly contributes to the oscillation of core clock gene transcript and this deadenylation process is controlled by PERIOD (Grima et al. 2019).

In mouse, genome-wide poly(A) adenylome analysis by microarray studies showed that 2.5% of transcripts expressed in the liver undergo circadian oscillation in poly(A) tail length. This rhythmicity in the poly(A) length variation contributes to the rhythmic translation and protein abundance (Kojima et al. 2012). In addition, the mRNA of the deadenylase Nocturnin is rhythmically expressed in various circadian clock gene-expressing tissues such as the SCN, pineal gland, liver, kidney and testis indicating the importance of Nocturnin as a potential circadian regulatory component (Wang et

al. 2001). It is likely that rhythmically expressing Nocturnin regulates the clock output pathway associated with metabolic regulation (Green et al. 2007). Although studies revealed the post-transcriptional contribution of circadian deadenylase Nocturnin in regulating the amplitude of metabolic pathways, it is worth to explore whether other deadenylases such as CNOT6/7/8 deadenylases of the mammalian CCR4–NOT complex play any role in posttranscriptional regulation of the core clock components (Stubblefield et al. 2018; Grima et al. 2019).

RNA binding proteins in circadian rhythm regulation

Accumulating evidence suggests that RNA binding proteins (RBP) and ribonucleoprotein (RNP) complexes regulate the expression of core clock genes by contributing to certain mRNA processing steps such as mRNA capping, splicing and intracellular transport of mRNA. A recent study reported the importance of the spliceosomal complex in the circadian regulation of *Drosophila* (Shakhmantsir et al. 2018). Spliceosome, RNA-protein complex is composed of five small nuclear ribonucleoprotein particles such as U1, U2, U4, U5, U6 snRNP and additional proteins associated with each snRNP complex (Wahl et al. 2009). RNA interference screen studies showed that pre-mRNA processing factor 4 (PRP4), a component of the U4/U5.U6 triple small nuclear ribonucleoprotein (tri-snRNP) complex and multiple tri-snRNP components regulate the molecular clock and free running periodicity. PRP4 contributes to the differential splicing of *tim* and thereby delays the accumulation of TIM in a 24h cycle (Shakhmantsir et al. 2018). This study shed light on a possible mechanism that delays the TIM protein accumulation in comparison to its mRNA oscillation. Another study reported the contribution of a novel gene *twenty-four* (*tyf*) in the lag between PER protein accumulation and mRNA in circadian pacemaker neurons. Mutants of *tyf* gene displayed weak behavioral rhythmicity associated with a reduction in the level of PER protein in *Drosophila* (Lim et al. 2011). TYF is associated with the 5′-cap binding complex, poly(A)-binding protein (PABP) and the clock gene *per* transcript, and it promotes the PER translation in pacemaker neurons in the fly brain to sustain the robust behavioral rhythmicity (Lim et al. 2011). Taken together, these studies decoded the importance of RBPs in post-transcriptional regulatory processes to delay the clock protein accumulation relative to that of its transcript oscillation.

A separate study deciphered the importance of RBP in clock output pathways. It was postulated that *Drosophila* RNA-binding protein LARK is required to transfer the temporal information from the molecular core clock to different output behavioral pathways through post-transcriptional repression of the distinct RNA targets (Newby and Jackson 1996; Schroeder et al. 2003; Huang et al. 2009). Biochemical and genetic analyses showed that LARK is associated with a large number of target RNA molecules and LARK selectively regulates the adult emergence rhythm by coordinating the temporal expression of Ecdysone induced protein-74 (E74) (Newby and Jackson 1996; Huang et al. 2007). While LARK regulates the output behavioral rhythm in *Drosophila,* it indeed acts as a post-transcriptional regulator of the mammalian circadian clock. In the mouse circadian pacemaker, LARK protein oscillates in phase with the PER1 protein. Further studies showed that LARK interacts with the *Per1* 3’UTR and post-transcriptionally regulates the expression of core clock component *Per1* (Kojima et al. 2007).

Studies in cultured fibroblasts showed that temperature cycles govern the rhythmic expression of cold-inducible RNA-binding protein (CIRP). CIRP maintains the robustness of the circadian timing system by binding to the transcripts of various core clock genes such as *Clock, Period 3, Rora* and clock associated gene *Sirtuin 1* (Morf et al. 2012). Further studies identified another novel post-transcriptional mechanism in the thermal entrainment of the circadian clock mediated via two cold-induced RBPs, Cirbp and Rbm3 in mouse (Liu et al. 2013). This study showed that Cirbp and Rbm3 control the high-amplitude oscillation of temperature entrained clock gene expression by regulating the alternative polyadenylation of the target genes. Furthermore, low temperature lengthened the 3'UTR and significantly upregulated Cirbp and Rbm3. It was found that these two RBPs repress the usage of proximal polyadenylation site (PAS) by binding to the 3'UTR sites. This study implied the potential function of Cirbp and Rbm3 in temperature entrainment of the circadian clock by regulating the alternative polyadenylation of core clock components such as *Bmal1*, *Clock*, *Per1* and *Cry1* (Liu et al. 2013).

RNA methylation and circadian rhythm

RNA methylation is a lesser known process compared to DNA or protein methylation. However, the importance of RNA methylation as a key post-transcriptional regulatory mechanism is being increasingly studied in recent years. A landmark study conducted in mice embryonic fibroblasts showed that global down regulation of RNA methylation by reducing the methylation potential of the cells resulted in increased circadian period (Fustin et al. 2013). A similar increase in period of the locomotor activity rest rhythm was observed when RNA methylation was down regulated in the SCN. Transcriptome analysis by RNA-Sequencing in the same study revealed that the transcription of the RNA processing machinery is altered by RNA methylation and RNA immunoprecipitation with anti-m6A antibody (MeRIP) showed that a number of circadian relevant transcripts such as *Per1, Per2, Per3, Clock, Arntl* have methylation sites on their transcripts (Fustin et al. 2013). A subsequent study showed that the RNA methylation levels in the mice liver RNA exhibit circadian rhythm and the *Cry 1/2*- knockout mice had significantly lowered levels of m6A on their mRNAs and the circadian rhythm of m6A methylation was lost in them compared to the wild type mice liver (Wang et al. 2015). These studies suggest that the relationship between the m6A methylation and circadian clock is bidirectional with the RNA methylation status affecting the speed of the circadian clock and the circadian clock in turn regulating the levels of RNA methylation in the liver transcripts.

Another study shows an important role of RNA methylation on the circadian regulation of hepatic lipid metabolism. This study showed that the deletion of hepatic *Bmal1* lead to altered expression and diurnal variations in the levels of methyl transferases, demethylases and methyl specific binding proteins and in turn lead to an increased mRNA methylation particularly of peroxisome proliferator-activator α (*PPaRα*), a major regulator of hepatic lipid metabolism (Zhong et al. 2018). The exact mechanism of how RNA methylation regulates the circadian clock or how it regulates the circadian control of metabolism is still unclear. However a recent study discusses some of the theoretically possible mechanisms by which RNA methylation can affect the period. This study suggests two possible mechanisms by which the decreased RNA methylation can result in period lengthening, either an

increased stabilization of non oscillatory mRNAs such as casein kinase 1δ (*CK1δ)* that phosphorylates clock component and a subsequent distortion of the circadian waveform or an increased stabilization of oscillatory mRNAs such as *Per* and *Cry* (Gibo and Kurosawa 2020). These studies suggest that RNA methylation is critical for circadian function and for circadian regulation of downstream genes.

microRNA mediated regulation of circadian rhythm

miRNA mediated post-transcriptional regulation has emerged as another major player in circadian timekeeping. miRNAs bind with the complementary target sites within the 3'UTR of selected mRNAs and down-regulate the gene expression through mRNA degradation or translational repression (Bartel 2004). Studies indicate that miRNAs regulate the circadian gene expression and play an important role in generating the time delay in circadian feedback inhibition, thereby fine-tune 24-hr circadian rhythmicity (Kadener et al. 2009; Chen et al. 2013; Chen et al. 2014). In the following section, we summarize the current understanding of the miRNA-mediated regulation of core clock machinery, circadian neural circuit excitability, clock input, output pathways and peripheral clocks based on the studies carried out in flies (Figure 1, Table 1) and mammals (Figure 2, Table 1).

Regulation of core clock machinery by miRNAs

To assess the role of miRNA in *Drosophila* core clock machinery, studies were conducted by impairing the miRNA biogenesis pathway and it severely dampened the activity-rest rhythms indicating the importance of miRNAs for maintaining robust circadian rhythmicity (Kadener et al. 2009). Subsequent studies used tiling array to identify the potential miRNAs that were responsible for maintaining the robustness of circadian rhythm, and it was found that 27 miRNAs are expressed in the circadian tissue (Kadener et al. 2009). Out of these 27 miRNAs, further biochemical experiments focused on *bantam* showed that evolutionarily conserved *bantam* binding target sites were located in the core clock component *Clk* 3'UTR (Kadener et al. 2009). Overall, the results of this study indicated the importance of miRNAs in circadian rhythmicity, especially *bantam* regulating the *Clk* expression in the circadian pacemaker to sustain the pace of the circadian clock (Kadener et al. 2009). Further studies identified *let-7* as a potential miRNA involved in the regulation of another core clock component in *Drosophila*. *let-7* regulates locomotor activity circadian rhythm through repression of the circadian gene *clockwork orange (cwo)* expression in the central clock (Chen et al. 2014). *cwo* is rhythmically expressed and contributes to the robustness of core clock gene oscillation (Lim et al. 2007; Kadener et al. 2007; Matsumoto et al. 2007). Studies also reported a third microRNA, miR-276a that regulates robust circadian behavioral and molecular rhythms by binding to a single target site in the 3'UTR of the core clock gene *tim* and suppress the expression of TIM in *Drosophila* (Chen and Rosbash 2016). Furthermore, a recent study based on the CLEAR-CLIP analysis showed that miR-375 modulates circadian rhythm and sleep by targeting *tim* in *Drosophila* (Xia et al. 2020). This study also identified thousands of miRNA-mRNA interactions and disruption of *Clk* affected this miRNA-mRNA interactome. In particular, the miRNA-mRNA circadian relevant interactions involving *tim*, *vri* and *pdp1* exhibited distinct changes in Clk^{Jrk} mutants. (Xia et al. 2020).

miRNAs that govern mammalian core clock timing mechanisms have also been identified. A forward genetic screen by using miRNA expression library in HeLA cells identified miR-192/194 gene cluster as a new mechanism for the post-transcriptional regulation of mammalian core clock machinery by negatively regulating the expression of the entire *Per* gene family, which consists of *Per1, Per2 and Per3* (Nagel et al. 2009). Subsequent studies carried out in NIH3T3 cells also yielded similar results where overexpression of mR-192/194 altered the circadian periodicity by regulating the expression of *Per* genes (Nagel et al. 2009). Another study conducted in HEK293 cells showed that co-expression miR-132 and miR-219 augmented CLOCK/BMAL1 dependent *Per1* transcription suggesting the role of these microRNAs in core clock timing mechanism by acting as the positive modulators of CLOCK/BMAL1 dependent *Per1* transcription (Cheng et al. 2007). TargetScanMouse algorithm was used in a successive study to identify other miRNAs targeting the 3'-UTR of *Per1* and *Per2* mRNA. This study identified the three most promising miRNAs miR-24, 29a and 30a that interact with the 3'-UTR of *Per1* and *Per2* mRNA to affect the stability and translation efficiency of these core clock gene mRNAs (Chen et al. 2013). Furthermore, this study used *Dicer* mutant and showed that miRNAs delay the cytoplasmic accumulation of PER and thus miRNAs are critical to generate time delay for the feedback loop inhibition of the circadian oscillator (Chen et al. 2013).

Another study on *Per2*::LUCIFERASE (*Per2*::LUC) circadian reporter mouse lines assessed the role of *Per2* 3'-UTR in the post-transcriptional regulation of circadian clock. The *Per2*::LUC reporter mice retained the endogenous 3'-UTR region of the *Per2* gene, whereas *Per2* 3′-UTR was replaced by an SV40 late poly(A) signal in *Per2*::LucSV mice. SCN and tissue explants from *Per2*::LucSV mice showed enhanced circadian bioluminescence level and oscillatory amplitude than the *Per2*::LUC reporter mice. Further molecular and genetic analysis showed that miR-24 binds to the *Per2* 3′-UTR to attenuate PER2 protein translation. This study provided important insights into the regulatory role of *Per2* 3′-UTR and miR-24 in core clock function (Yoo et al. 2017). A recent study used *in silico* algorithms and found that the novel miRNA-25 fine-tunes the circadian rhythmicity by repressing *Per2* expression (Park et al. 2020). Luciferase reporter assays validated the predicted binding sites of miR-25 in the 3' UTR of *Per2* mRNA (Park et al. 2020). One more recent study used a genome-wide miRNA screen and identified miRNA cluster miR-183/96/182 that modulates circadian rhythms. Potential targets of this miRNA cluster were predicted by using computational methods miRanda and DIANA-microT-CDS. Both the methods predicted and luciferase reporter assay validated *Per2* as the direct target of miR-96 (Zhou et al. 2021). In summary, these studies elucidated the role of miR-192/194, miR-132, miR-219, miR-24, miR-29a, miR-30a, miR-25 and miR-96 in post-transcriptional regulation of circadian rhythm by regulating the expression of the entire *Per* gene family.

miRNAs are also known to regulate various integral components of the mammalian core clock machinery other than the *per*. The pre-miRNA overexpression constructs of miR-494 and miR-142-3p in HEK293 cells and SCN cells repressed *Bmal1* 3′ UTR activity indicating the importance of these microRNAs as the posttranscriptional regulators of core clock element *Bmal1* (Shende et al. 2011; Shende et al. 2013). Furthermore, studies showed that miR-17-5p is rhythmically expressed in SCN and inhibits the translation of *Clock* by binding the 3'UTR of its mRNA and shorten the circadian period. This study

demonstrated CRY1 as an important output molecule for miR-17-5p mediated stabilization of the circadian period (Gao et al. 2016).

Regulation of clock neural circuit morphology and excitability

In *Drosophila*, approximately 150 neurons in the brain are important for orchestrating the rhythmicity in behavior. This circadian neuronal network consists of subsets of dorsal neurons (DNs), dorsal lateral neurons (LNd), small and large ventral lateral neurons (sLNv and lLNv), and lateral posterior neurons (LPN) (Helfrich-Förster 1997; Kaneko et al. 1997; Kaneko and Hall 2000; Klarsfeld 2004; Shafer et al. 2006). Four of the five sLNvs and all the lLNvs express circadian neuropeptide Pigment dispersing factor (PDF) (Shafer et al. 2008). PDF cells mediate the circadian period and the PDF cell termini undergo circadian morphological changes during the daily fasciculation and defasciculation remodelling cycles (Fernández et al. 2008; Guo et al. 2014). A growing body of evidence suggests that miRNAs regulate neuronal activity and synaptic plasticity in *Drosophila* and mammals (Tan et al. 2013; Verma et al. 2015). Extensive studies by using ArcLight, GCaMP6 imaging and *In vivo* calcium monitoring showed that miR-92a suppresses PDF neuronal excitability in flies. Adult stage-specific manipulation of miR-92a levels altered the fasciculation-defasciculation state of the PDF cell termini. Results obtained from translating ribosome affinity purification (TRAP) and miRNA target prediction tool Targetscan indicated that miR-92a suppresses neuronal excitability partly by targeting *sirtuin 2 (sirt2*) in PDF cells (Chen and Rosbash 2017).

A consecutive study investigated the effect of misexpressing miR-210 in circadian behavior and on circadian clock neurons. Overexpression of miR-210 altered the projections and morphology of the PDF expressing clock neurons. It also increased PER levels without affecting it's cycling in the clock neurons and modulated the phase of activity-rest rhythm (Cusumano et al. 2018). miR-263b was also identified as an important regulator of circadian rhythm and structural plasticity of PDF positive sLNv axonal projections. *In silico* prediction algorithm was used to identify the potential targets of miR-263b and the results of subsequent genetic analysis showed that miR-263b regulates the arborisation rhythms of sLNv dorsal projections and activity-rest rhythm by suppressing the expression of LIM-only protein *Beadex* (*Bx*) (Nian et al. 2019).

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus functions as the central circadian pacemaker. In addition to directing the daily behavioral rhythm, it senses changes in day length and seasonal adaptation relies predominantly on the structural plasticity of SCN neuronal network (Meijer et al. 2010). miR-132/212 modulates entrainment to various photoperiods and day length mediated regulation of PER2 expression in the SCN (Mendoza-Viveros et al. 2017). Proteomic analysis also showed that miR-132/212 play a key role in regulating the seasonal changes in the dendritic morphology of circadian pacemaker neurons. miR-132/212 mediate this dendritic morphology changes through the direct target, methyl CpG binding protein 2 (MeCP2) dependent mechanism and thus enables the SCN to encode photoperiod changes (Mendoza-Viveros et al. 2017).

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Regulation of clock input pathways

In *Drosophila*, no miRNA has been implicated in the regulation of circadian input pathways yet. Whereas in mammals, miR-132 has been implicated in the photic input pathways of the circadian clock. Knockdown of miR-132 in the SCN enhanced the light-induced resetting effects of the clock and overexpression attenuated the clock resetting effects of light indicating that miR-132 acts as a negative regulator of photic entrainment (Cheng et al. 2007). A follow-up study used *in silico* analysis and *in vitro* 3' UTR assays to reveal the potential targets of miR-132. miR-132 regulates the expression of chromatin remodelling related target genes such as *Mecp2*, *Jarid1a* and *Ep300*. This study collectively showed that miR-132 mediates the circadian clock entrainment through its downstream targets involved in chromatin function and protein translation within SCN (Alvarez-Saavedra et al. 2011). In the chicken retina, miR-26a exhibits diurnal oscillation regulated by CLOCK. miR-26a drive circadian rhythm in the production of the L-type voltage-gated calcium channel α1C subunit in chicken cone photoreceptors (Shi et al. 2009). Given that L-type calcium channels play a central role in rhythmic melatonin synthesis, miR-26a could play an important role in circadian retinal physiology and light input (Ivanova and Iuvone 2003)

Regulation of clock output and peripheral oscillators

In addition to regulating the core clock machinery and the clock neuron morphology, microRNAs are important regulators of various clock output pathways. In *Drosophila*, miR-279 governs the activity-rest rhythm behavioral output without affecting the central clock. Further studies by using computational algorithms such as PicTar, TargetScanFly5.1, MiRanda and RNAi knockdown studies on transgenic flies revealed that miR-279 target *Unpaired* (*Upd*), the ligand for JAK/STAT pathway regulates the activity-rest rhythm in *Drosophila* (Luo and Sehgal 2012). In addition, miR-124 has a striking impact on the phase of circadian locomotor activity rhythm. Flies lacking miR-124 advanced the phase of activity-rest rhythm under constant darkness without altering the phase of the circadian pacemaker in the brain that controls this behavioral rhythm. This indicates that miR-124 shapes the diurnal activity and determines the phase of the behavioral output without affecting the central clock (Zhang et al. 2016). A recent study identified miR-263a as an important regulator of activity-rest rhythm. Downregulation of miR-263a in the clock neurons reduced the morning peak of activity and the robustness of the circadian rhythm. Moreover, overexpression of miR-263a in the clock neurons lengthened the periodicity. Further *in-vitro* assays showed that miR-263a suppress the potential target *slo* that encodes the structural alpha subunit of a BK calcium-activated potassium channel and activates *homer* that acts as an adaptor binding to the group I metabotropic glutamates and other intracellular signaling proteins (Nian et al. 2020). Both *slo* and *homer* contribute to the circadian locomotor activity (Jepson et al. 2012; Naidoo et al. 2012).

While miR-279, miR-124 and miR-263a regulate activity-rest rhythm without affecting the central clock, let-7 regulate locomotor activity behavioral output through the circadian gene *cwo* in the central pacemaker (Chen et al. 2014). miR-92a modulates neuronal excitability of PDF cells and this regulation is not only restricted to PDF cells. miR-92a regulates sleep and phase shift response through the mRNA target *sirt2* expressed in the dopaminergic neurons and PDF cells respectively (Chen and Rosbash 2017). Furthermore, miR-210 regulates the

phase of activity rest rhythm through repression of the cell adhesion molecule *Fasciclin 2* (*Fas 2*) (Niu et al. 2019). miR-210 modulates the morphology of clock neurons and the levels of PER without affecting the phase of its oscillation indicating that miR-210 plays a vital role in the circadian output pathways (Cusumano et al. 2018).

Accumulating evidence from various studies suggest that glial specific vesicle trafficking and release factors modulate the circadian rhythmic behavior (Ng et al. 2011; Ng and Jackson 2015; Ng et al. 2016). Glial secretion also modulates the PDF level within the s-LNv clock neuron projections indicating the importance of glial-neuronal communication in circadian rhythmicity (Ng et al. 2011). Studies addressed whether microRNAs expressed in astrocytes regulate circadian rhythmicity. The results of these studies showed that astrocyte-specific genetic inhibition of miR-263b and miR-274 in adult flies impedes the activity-rest rhythm without affecting the molecular clock and clock neuron viability (You et al. 2018). *In silico* procedures and genetic screens were used to identify the potential RNA targets of these microRNAs and genetic inhibition of miR-274 targets *CG4328* (a predicted LIM-homeodomain transcription factor) and *MESK2* (involved in Ras/ERK signaling and has homology with *N-myc downstream-regulated gene 2*) resulted in significantly reduced rhythmicity. This genetic screen identified the putative RNA targets of miR-274 in glia that might regulate circadian locomotor rhythm in *Drosophila* (You et al. 2018).

Apart from regulating the activity-rest rhythm, microRNAs also regulate other behavioral outputs. For example, a cluster of six microRNAs miR-959-964 undergo robust circadian cycling with a peak at ~ZT12 or close to lights off. Analysis of this cluster knock out and overexpression showed that these miRNAs impact circadian outputs such as innate immunity, metabolism, and feeding time. The circadian clock mediated timing of food intake in turn regulates the cluster miRNA cycling. These observations indicate that post-transcriptional feedback of cycling cluster miRNAs is involved in the timing of feeding (Vodala et al. 2012).

In mammals, studies were focused on the role of miRNA in peripheral clock tissues that drives the rhythm in physiological outputs such as lipid metabolism in the liver and enterocyte proliferation in the intestine. For instance, miR-122 was identified as a potential candidate that might regulate the circadian gene expression in the murine liver. miR-122 depletion resulted in upregulation of several circadian output genes specifically in the liver and had a striking effect on the circadian phase and amplitude of *Smarcd1/Baf60a* mRNA (Gatfield et al. 2009). SMARCD1/BAF60a is a component of the SWI/SNF chromatin-remodeling complex that regulates hepatic lipid metabolizing genes (Li et al. 2008). 3’ UTR assays confirmed peroxisome proliferator-activated receptor β/δ (*Pparβ/δ*) and the PPARα coactivator *Smarcd1/Baf60a* as the novel targets of miR-122. These results suggest the involvement of miR-122 in metabolic control via the metabolic regulators of the PPAR family (Gatfield et al. 2009). Additionally, a subsequent study showed that knockdown of miR-122 in the liver increased the expression and amplitude of Nocturnin rhythms (Kojima et al. 2010). Nocturnin is a circadian deadenylase enzyme that removes poly (A) tail from mRNAs and Nocturnin knockout mice have severe deficits in lipid metabolism (Green et al. 2007). These results suggest miR-122 as an important regulator of lipid metabolism by regulating the Nocturnin and PPAR family gene expression in the liver (Gatfield et

al. 2009; Kojima et al. 2010). In addition to regulating the physiological output in the liver, miRNAs govern the circadian control of intestinal physiology. For example, circadian oscillators synchronize the enterocyte proliferation and miR-16 is expressed rhythmically in the intestinal crypts. miR-16 is anti-proliferative in the enterocytes and it may act as an important link in the circadian control of intestinal proliferation rhythm (Balakrishnan et al. 2010).

Extracellular vesicles are excellent candidates as transmitters of signals between the central and peripheral oscillators and among various peripheral clocks (Tao and Guo 2018). miRNA- 494, miR- 152 and miR-142-3p are reported to be present in the mice serum and among them, miR-494 and miR-152 exhibit diurnal oscillations with bimodal peak of expression (Shende et al. 2011). Studies examined the role of extracellular miRNAs miR-142-3p and miR-494 in mediating the rhythmicity among peripheral circadian oscillators. Overexpression of these microRNAs decreased the endogenous level of BMAL1 protein by modulating the 3'UTR activity of its mRNA in cultured fibroblasts (Shende et al. 2013). In fact, a careful look at the online database of miRNA composition of microvesicles (http://microvesicles.org/Archive/VESICLEPEDIA_MIRNA_DETAILS_4.1.txt) reveals that miRNAs such as miR-312, miR-142-3p and miR-494 that are implicated in circadian rhythm are enriched in the microvesicles. miR-142-3p communicate among cells by vesicular trafficking and inhibition of this vesicle trafficking modulated the ensemble clock gene rhythms. A recent study in human adipocytes treated with the exosomes containing plasma from night shift workers showed that circadian misalignment is conveyed to the peripheral tissues through circulating exosomal miRNAs and it plays an important role in metabolic dysfunction (Khalyfa et al., 2020). These results suggest that vesicle trafficking of miRNAs may act as extracellular regulatory signals of time for coordinating autonomous circadian oscillators in peripheral tissues (Shende et al. 2011; Shende et al. 2013; Shende et al., 2014).

II. Circadian regulation of microRNAs

While miRNA-mediated post-transcriptional regulation fine-tunes the circadian oscillations, the circadian timekeeping system, in turn, drives the diurnal rhythm in the expression of a number of miRNAs. For example, microarray analysis of miRNAs in wild type and cyc^{0} mutant fly heads showed that miR-263a and miR-263b exhibit robust diurnal changes in abundance with a peak during the night in wild type flies. This rhythm persisted even under constant darkness in wild type flies whereas it was abolished in cyc^{0} flies indicating the circadian regulation of these miRNAs (Yang et al. 2008). Both miR-263a and miR-263b are predicted to target *Clk*. Other clock relevant potential targets of miR-263a and miR-263b include *doubletime* (*dbt*) and *cwo,* respectively. The results of this study suggest that cycling miRNAs play a fundamental role in the circadian system by fine-tuning the daily changes in mRNA and protein levels. A subsequent study sequenced fly head RNA to identify miRNAs that exhibit robust circadian oscillations. A cluster of six miRNAs, miR-959-964 exhibited high amplitude circadian oscillation with a peak close to lights-off under the light-dark (LD) cycle and this rhythm was disrupted in Clk^{Jrk} and per^{0} clock mutants (Vodala et al. 2012). This study showed that the circadian clock regulates the timing of food intake, which in turn regulates the cluster miRNA cycling. These miRNA oscillation timings modulate the mRNA translation associated with the cycling of multiple physiological processes including

the timing of feeding. Thus cycling cluster miRNAs contribute to a feedback circuit involved in the timing of feeding. (Vodala et al. 2012).

A successive study quantified the expression of 16 miRNAs in PDF- pacemaker neurons by using reverse transcriptase quantitative PCR (RT–qPCR) and among them, six microRNAs: miR-184, miR-276a, miR-999, miR-981, miR-210 and miR-92 exhibited cycling expression levels under LD cycles in wild type flies (Chen and Rosbash 2017). Among these six cyclers, only miR-92 oscillates with a peak during the night and circadian oscillations in mir-92a rhythmically regulate its functional target *sirt2*. SIRT2 levels expressed in a rhythmic manner govern the circadian fasciculation–defasciculation cycles in the PDF cell termini (Chen and Rosbash 2017). Circadian remodeling in the PDF cell termini propagates the time of the day information to the downstream targets underlying the activity-rest rhythm (Fernández et al., 2008). miR-210 exhibits rhythmic expression in the sLNvs and miR-210 regulates the timing of evening activity by inhibiting *Fas 2* in the optic lobe (Chen and Rosbash 2017; Niu et al. 2019). miR-276a expression level oscillates under LD cycles and not under constant darkness (DD) and it appears that the expression is light-regulated (Chen and Rosbash 2016). miRNA *let-7*, a component of the *let-7*-complex was identified as the circadian rhythm regulator and the expression level of *let-7* exhibited circadian oscillation with a peak during the day. Oscillation of *let-7* is indirectly regulated by the CLK/CYC through a pathway involving circadian prothoracicotropic hormone (PTTH) and CLK mediated Ecdysone receptor (EcR) signaling (Chen et al. 2014). Rhythmically expressed *let-7* regulates the amplitude of circadian oscillation by repressing the target gene *cwo* in the central oscillator (Chen et al. 2014).

In the murine brain, miR-219 exhibits robust circadian rhythm in expression with a peak during the subjective day and CLOCK/BMAL1 heterodimer regulates miR-219 expression. miR-219 plays a significant role in determining the circadian period and phase length (Cheng et al. 2007). PH domain and leucine rich repeat protein phosphatase (SCOP), a potential target of miR-219 is rhythmically expressed in the SCN with a peak during the subjective night and SCOP may be a physiologically relevant target of miR-219 within the SCN that mediate the effect of miR-219 on clock timing (Shimizu et al., 1999; Cheng et al. 2007). In addition, brain-specific miRNA miR-132 displays a modest significant rhythm with a peak during the subjective day and the expression of miR-132 is regulated by light and cAMP response element-binding protein (CREB). miR-132 acts as a negative regulator of the light-induced clock resetting (Cheng et al. 2007). The expression of putative miR-132 target regulatory factor X4 (RFX4), a member of the winged subfamily of helix-turn-helix transcription factors is light inducible and it is abundantly expressed in the SCN (Araki et al., 2004). Based on the in-vitro overexpression studies for target validation and by considering the light inducible nature of both miR-132 and RFX4, it is hypothesised that RFX4 may be the physiologically relevant target in the SCN that mediate the effect of miR-132 on clock entrainment (Cheng et al. 2007).

Furthermore, miR-17-5p is rhythmically expressed in mouse SCN implicating that its expression is under the circadian clock control (Gao et al. 2016). The expression rhythm of miR-17-5p and CLK was antiphasic with similar oscillatory periods and chromatin immunoprecipitation assay showed that CLOCK directly binds to the promoter of miR-17

and regulates its expression (Gao et al. 2016). miR-17-5p acts as a modulator of the circadian rhythm periodicity by interacting with *Clock* and potentially through CRY1 output molecule. In both flies and mammals, a small proportion of microRNAs exhibit clock regulated circadian rhythm in expression and it is likely that the rhythmic expression of miRNAs fine-tunes the level of specific proteins that generate robust circadian oscillations. These studies also suggest that circadian clock driven miRNA rhythmicity regulates the cyclic expression of their potential targets associated with diverse physiological functions and thus acts as critical mediators of rhythmic physiological processes.

Role of circadian relevant miRNAs in diseases

A number of disease conditions are known to have certain circadian misalignments and miRNA dysregulation as the underlying cause for disease onset and progression. Several studies have shown the involvement of circadian relevant miRNAs in cancer ontology. For example, downregulation of *Per3* by miR-103 is observed in colorectal cancer cells and pancreatic clock disruption by the action of miR-135b on *BMAL1* contributes to pancreatic cancer progression (Hong et al. 2014; Jiang et al. 2018). NPAS2, a core clock component is an oncogene in hepatocellular carcinogenesis and is up regulated in the Hepatocellular carcinoma cells due to the down regulation of miR-199b-5p (Yuan et al. 2017; Yuan et al. 2020). Another study shows the role of miR-206 targeting of *Clock* in glioma progression (Huang et al. 2019).

Altered expression of miRNAs is implicated in several sleep and circadian disorders. A study conducted in mice showed that the expression of nearly 50 miRNAs was affected following sleep loss (Davis et al. 2007). Another study showed that the REM sleep deprivation changes the hippocampal expression of miR-132, miR-182 and miR-124 in mice (Karabulut et al. 2019). In humans, the levels of circulating miR-125a, miR-126 and miR-146a were significantly lowered in habitual short sleep group compared to normal sleep group (Hijmans et al. 2019). Fatal familial insomnia shows significant association with a Single Nucleotide Polymorphism (SNP) in miR-146a and polymorphism in the pre-miR-182, a known circadian clock regulator, shows association with late insomnia in major depression patients (Saus et al. 2010; Gao et al. 2018). A study that was conducted in narcolepsy patients demonstrated that the levels of four miRNAs viz; miR-30c, let-7f, miR-26a and miR-130a were altered in the plasma compared to the controls (Holm et al. 2014).

Rhythmically expressed miRNAs are also associated with the disease progression of certain neurodegenerative diseases such as Alzheimer's disease (AD) and Huntington's disease (HD). For instance, miR-210 is rhythmically expressed in clock neurons and is known to regulate the phase circadian locomotor rhythms in *Drosophila* (Niu et al. 2019). miR-210 is evolutionarily conserved between *Drosophila* and mammals (Weigelt et al. 2019). It is also validated that miR-210 regulates various target genes involved in neurodegenerative pathways of Alzheimer's, and Huntington's diseases. The dysregulation of miR-210 in various brain tissues and cerebrospinal fluid has also been correlated with the pathology of Alzheimer's disease (Watts et al. 2018). miR-146a shows rhythmic expression and is up regulated in various brain regions of AD patients and this miRNA has been suggested to

play a role in the pathogenesis of AD by regulating the genes involved in cerebrovascular dysfunction and neuroinflammation (Wang et al. 2014; Swarbrick et al. 2019). miRNAs such as miR-132 and miR-124 are associated with Huntington's disease. Photic entrainment cues induce the expression of miR-132 in mice and its expression is altered in the brains of HD patients (Cheng et al. 2007; Hoss et al. 2015). miR-124 is conserved across the animal kingdom and it is down-regulated in the brains of HD patients (Johnson et al. 2008; Xue et al. 2016). miR-124 is rhythmically expressed and regulates the circadian output in flies (Yang et al. 2008; Zhang et al. 2016). These results suggest that progression of various neurodegenerative diseases are linked to circadian rhythm and miRNA dysregulation.

Perspectives and conclusion

Accumulating evidence from various studies have shown that transcriptional-translational feedback loop regulation and post-translational regulation play a major role in circadian rhythmicity. Recent studies also showed that microRNA mediated post-transcriptional regulation is concomitantly important in shaping rhythmic gene expression. In this review, we summarized the importance of various post-transcriptional mechanisms such as splicing, polyadenylation, RNA binding proteins, RNA methylation and microRNAs in generating circadian rhythmicity of mRNAs and ultimately the rhythmic behavior and physiology of an organism. miR-132 and miR-26a are the only miRNAs that have been implicated in the photic input pathways of the circadian clock. The current understanding of miRNA mediated post-transcriptional regulation of clock input pathways is still rudimentary. It is possible that in addition to miR-132 and miR-26a, other microRNAs are involved in the clock input pathways and further characterization of miRNAs involved in the light, temperature and food entrainment will provide novel insights into the post-transcriptional control of the circadian clock input pathways.

miRNAs not only regulate the timekeeping function of the central clock, it also impacts the rhythmic clock gene expression in the peripheral tissues (Na et al. 2009; Shende et al. 2014). In addition, miRNAs are rhythmically expressed in both the central clock and in multiple peripheral tissues (Na et al. 2009; Balakrishnan et al. 2010). A previous study showed that miR-433 regulates glucocorticoid signaling and thus helps to maintain circadian rhythm in osteoblasts (Smith et al. 2016). Although studies indicate the importance of miRNAs in peripheral circadian rhythmicity, the broad impact of miRNAs in the coupling between the central and peripheral clocks present in various tissues remains to be explored.

In this review, we also discussed the specific role of miRNAs in regulating the core clock machinery, circadian neural circuit excitability, clock input and output pathways. Only a handful of miRNAs have been reported to be vital for circadian clock function. Apart from this, uncharacterized microRNAs may also play a key role in maintaining the circadian rhythm. Extensive future studies on novel circadian relevant miRNAs will provide insights into the post-transcriptional regulation of the circadian clock by non-coding RNAs. In addition to miRNA mediated post-transcriptional regulation, alternative splicing is currently recognized as an additional layer of regulation at the clock gene pre-mRNA level. However a comprehensive understanding of the physiological importance of splicing events of various clock pre-mRNAs including *Cry1* and *Per2* in mammals remains elusive. We

summarized the current understanding on RBPs that regulate the core clock gene expression by modulating the mRNA processing steps. Apart from these evidence on RBPs regulating the circadian phenotypes, it is important to identify the remaining RBPs associated with circadian clocks and their functional relevance to provide valuable insights into the post-transcriptional regulation of the circadian timekeeping system.

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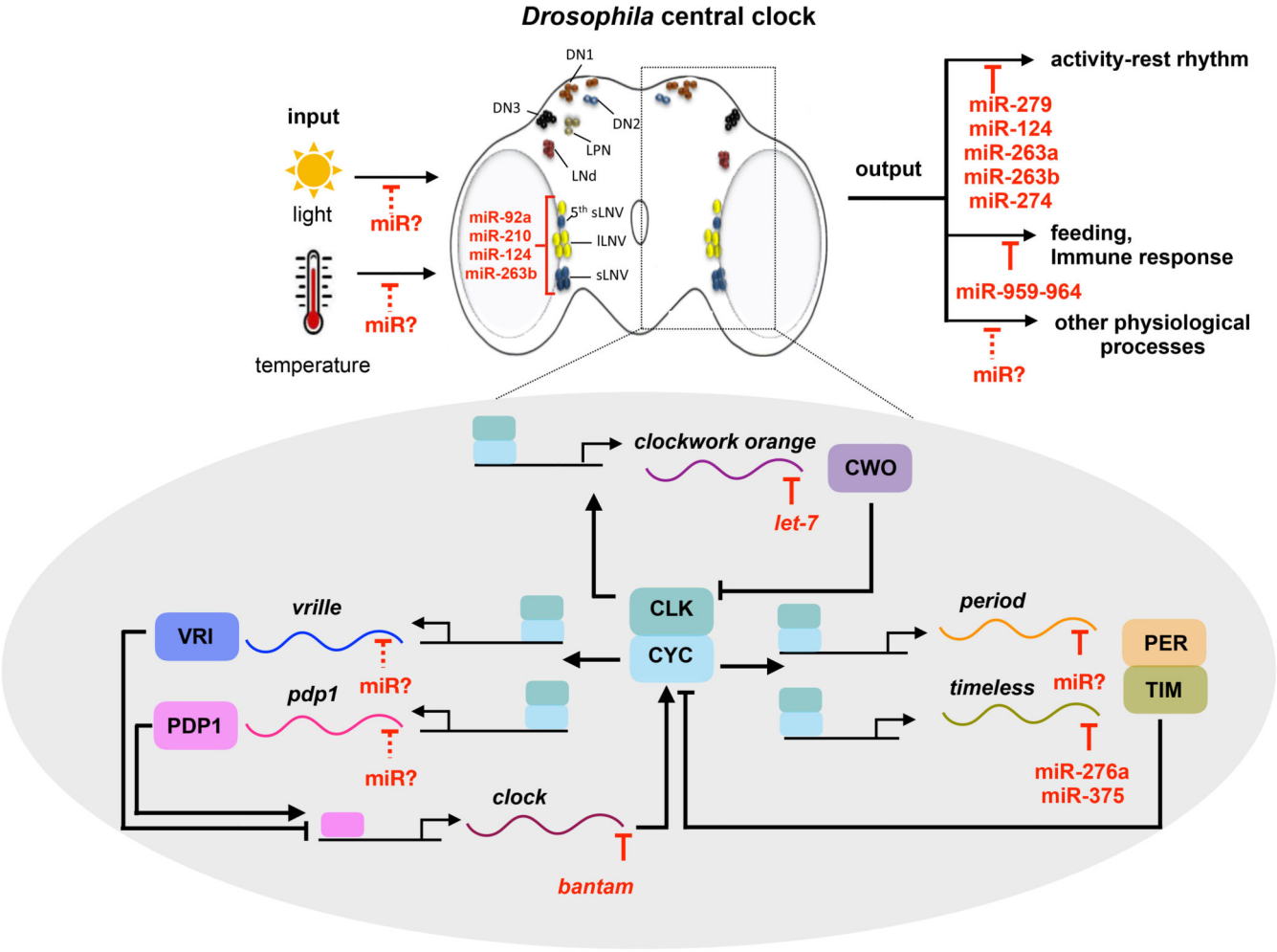

Figure 1. microRNA mediated regulation of circadian rhythm in *Drosophila*

Circadian timing system is depicted with three primary components such as input pathways, the central oscillator and the output pathways. Environmental cues such as light and temperature entrain the central oscillator. Central oscillator shown in the schematic cross section of the fly brain comprises of ~150 clock neurons and it consists of large ventrolateral neurons (l-LNvs, yellow), Small ventrolateral neurons (s-LNvs, blue), lateral posterior neuron (LPN- green), dorsolateral neurons (LNds, red), and dorsal neurons (DN1- orange, DN2-blue open circle, DN3-black). The endogenous rhythmicity of the central oscillator is driven by interlocked primary and secondary transcriptional-translational feedback loop. The primary feedback loop consists of positive limb component CLK/CYC and a negative limb PER/TIM. VRI and PDP1 in the secondary feedback loop respectively inhibits and activates the transcription of *Clk.* In addition, the transcription repressor CWO inhibits the transcription of *per* and *tim* by competing with CLK-CYC for the E-box binding. Several microRNAs regulate the core clock components, clock neuron excitability, input and output pathways of *Drosophila* circadian timing system. Blunt end lines denote inhibitory effects. Solid and dashed lines denote known and putative pathways respectively.

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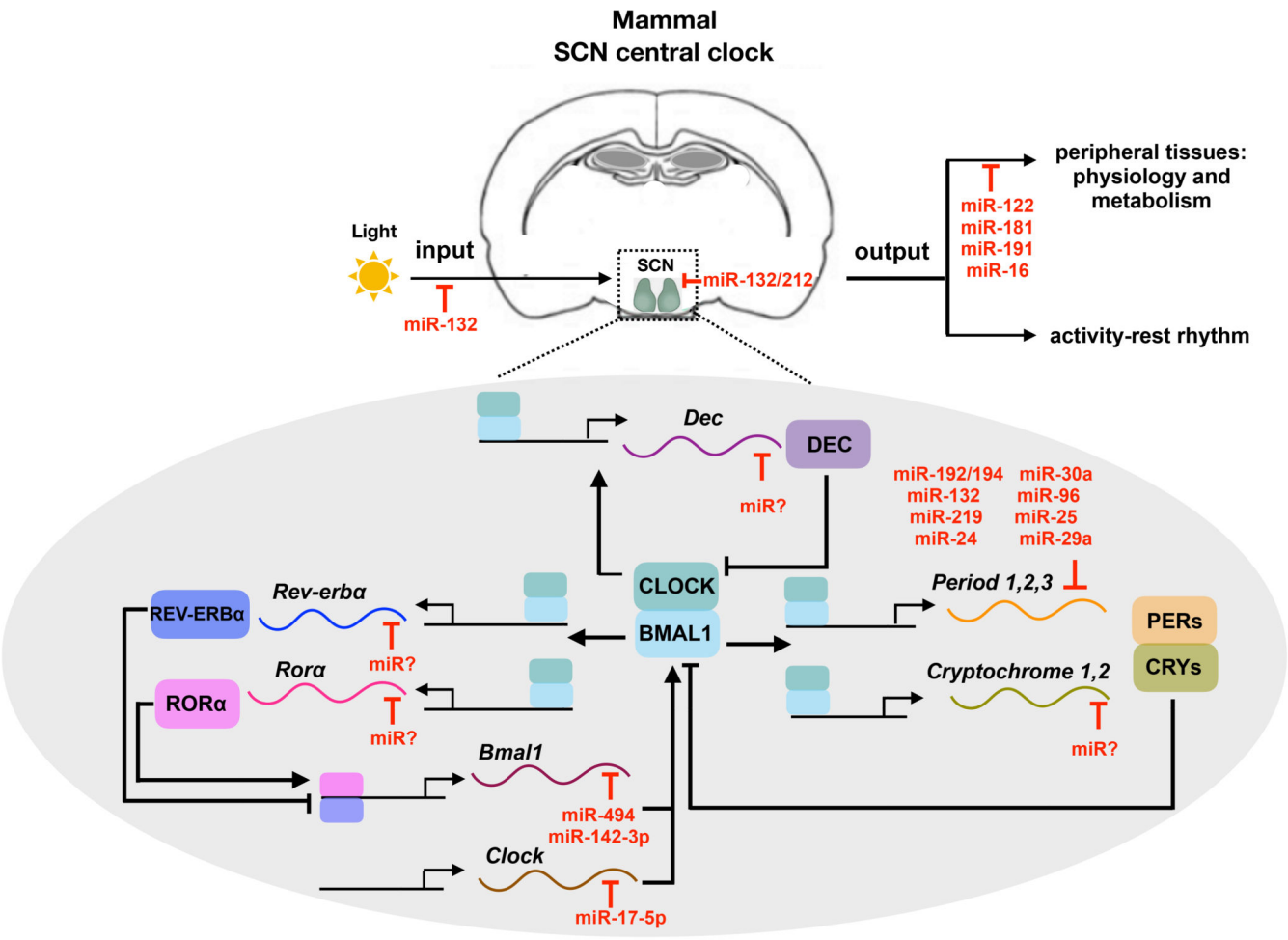

Figure 2. microRNA mediated regulation of circadian rhythm in mammals

In mammals, light input is conveyed to the central oscillator suprachiasmatic nucleus (SCN). SCN is located at the base of the hypothalamus as shown in the schematic cross section of the adult mammalian brain. Primary and secondary feedback loops operating in the SCN neurons generates ~24 hour rhythmicity in behaviour and physiological outputs in the peripheral tissues. The primary TTFL consists of the negative arm PER/CRY and a positive arm CLOCK/BMAL1. The secondary feedback loop components REV-ERB and ROR repress and activate the *Bmal1* transcription respectively. Furthermore *Differentiated embryo chondrocyte (Dec)* transcription factor interacts with *Bmal1* and inhibits CLOCK/BMAL1 induced transcription. Various microRNAs regulate the core clock components, clock neuron morphology, input and output pathways of mammalian clock system. Blunt end lines denote inhibitory effects. Solid and dashed lines denote known and putative pathways respectively.

Table 1
miRNAs involved in circadian regulation.

List of miRNAs present in *Drosophila* and mammals that are involved in regulating different aspects of the circadian clock. The list includes the targets of miRNAs and the target validation techniques used.

	Organism	microRNA	Target	Target validation technique used
Core clock machinery	*Drosophila*	*bantam*	*Clk* (Kadener et al. 2009)	Luciferase Reporter Assay
		let-7	*cwo* (Chen et al.2014)	Luciferase Reporter Assay
		miR-276a	*tim* (Chen and Rosbash 2016)	Luciferase Reporter Assay
		miR-375	*tim* (Xia et al. 2020)	qRT-PCR and Western Blot
	Mammals	miR-132	(Cheng et al. 2007)	
		miR-219	(Cheng et al. 2007)	
		miR-192/194 cluster	*Per1,Per2,Per3*(Nagel et al. 2009)	Luciferase Reporter Assay
		miR-494	*Bmal1* (Shende et al. 2011)	Luciferase Reporter Assay
		miR-142-3p	*Bmal1* (Shende et al. 2011; Shende et al. 2013)	Luciferase Reporter Assay
		miR-24	*Per1* (Chen et al. 2013), *Per2*(Chen et al. 2013;Yoo et al. 2017;Park et al. 2020)	Luciferase Reporter Assay
		miR-30a	*Per1* (Chen et al. 2013)	Luciferase Reporter Assay
		miR-29a	*Per1* (Chen et al. 2013)	Luciferase Reporter Assay
		miR-17-5p	*Clock* (Gao et al. 2016)	Luciferase Reporter Assay
		miR-25	*Per2* (Park et al. 2020)	Luciferase Reporter Assay
		miR-96	*Per2* (Zhou et al. 2021)	Luciferase Reporter Assay
Clock neuronal circuitry	*Drosophila*	miR-92a	*sirt2* (Chen and Rosbash 2017)	Luciferase Reporter Assay
		miR-210	(Cusumao et al. 2018)	
		miR-263b	*Bx*(Nian et al. 2019)	Luciferase Reporter Assay
	Mammals	miR-132/212	*MeCP2* (Mendoza-Viveros et al. 2017)	Phenotypic Rescue Experiment
Clock input pathway	Mammals	miR-132	*MeCP2, Jarid1a, Ep300,Btg2,Paip2a* (Alvarez-Saavedra et al. 2011)	Luciferase Reporter Assay
Clock output pathway and peripheral tissues	*Drosophila*	miR-279	*Upd* (Luo and Sehgal 2012)	Luciferase Reporter Assay
		miR-959-964 Cluster	(Vodala et al. 2012)	
		miR-124	(Zhang et al 2016)	
		miR-274	*MESK2, CG4328* (You et al. 2018)	Phenotype study using RNAi line

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	Organism	microRNA	Target	Target validation technique used
		miR-263b	You et al.(2018)	
		miR-210	*Fas2*(Niu et al. 2019)	Phenotype study in mutated Fas2 3'UTR flies
		miR-263a	*slo, homer*(Nian et al 2020)	Luciferase Reporter Assay
	Mammals	miR-122	*Pparβ/δ, Smarcd1/Baf60a* (Gatfield et al. 2009), *Nocturnin* (Kojima et al. 2010)	Luciferase Reporter Assay
		miR-16	(Balakrishnan et al. 2010)	