Timing Without Coding: How do long non-coding RNAs regulate circadian rhythms?

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Abstract

Long non-coding RNAs (lncRNAs) are a new class of regulatory RNAs that play important roles in disease development and a variety of biological processes. Recent studies have underscored the importance of lncRNAs in the circadian clock system and demonstrated that lncRNAs regulate core clock genes and the core clock machinery in mammals. In this review, we provide an overview of our current understanding of how lncRNAs regulate the circadian clock without coding a protein. We also offer additional insights into the challenges in understanding the functions of lncRNAs and other unresolved questions in the field. We do not cover other regulatory ncRNAs even though they also play important roles; readers are highly encouraged to refer to other excellent reviews on this topic.

Keywords

Long non-coding RNA, antisense transcript, circadian rhythms, molecular clock

1. Introduction

A central dogma in biology describes that genetic information embedded in DNA is passed on to a protein, whereas RNA is simply an intermediate molecule that transfers genetic information. Therefore, it came as a surprise to find that up to 80% of genomes are actively transcribed, yet, only about 2% of the genome is used to encode a protein in humans [1, 2]. Transcripts that do not have the potential to encode a protein are called "non-coding" RNAs (ncRNAs), and are considered to be particularly important for the complexity of the organism as the ratio of ncRNA to total genome size significantly increases in higher eukaryotes [3, 4]. Some ncRNAs have been well-characterized and have been shown to exert important functions. For example, rRNA and tRNA are required for mRNA translation, while small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) are essential for splicing and RNA modification, respectively [5, 6]. New classes of ncRNAs, such as microRNAs (miRNAs), enhancer RNAs (eRNAs), and circular RNAs (circRNAs), also have a variety of regulatory roles [5, 6]. These ncRNAs can be divided into short- or long ncRNAs (lncRNAs) using the arbitrary cutoff of 200 nt [7].

LncRNAs can be categorized by their genomic location and orientation in relation to nearby protein coding genes [8, 9] (Figure 1). They were originally considered background transcription noise and to lack defined functions, mainly because of their low expression level and the lack of conservation in their primary sequences [10-14]. More recent studies, however, discovered conservation in their genomic positions (synteny), promoter sequence, and secondary structure, suggesting that lncRNAs carry important genetic information without relying solely on their sequence [13-15]. In fact, studies in the past decade have found that lncRNAs elicit a wide spectrum of functions in diverse biological processes such as X inactivation, cell differentiation,

and neuronal or immune functions as well as disease development including cancer, neurodegeneration, and congenital genetic diseases [16, 17].

Recent studies have also underscored the importance of lncRNAs in regulating circadian rhythms. Although it is still in its infancy, some studies found hundreds of lncRNAs whose expression is either rhythmic or induced by the activation of cyclic AMP (cAMP) pathway, implicating their functions in the circadian clock system (Table 1) [18-21]. Other studies found a more direct link between lncRNAs and the circadian clock and demonstrated that lncRNAs regulate core clock genes and the core machinery (Table 2). In this review, we discuss: 1) How is the transcription of lncRNA regulated?, 2) How can lncRNAs regulate target genes without encoding a protein?, and 3) Are the functions of lncRNAs in the circadian clock system evolutionarily conserved?, while highlighting the differences and similarities between lncRNAs and mRNAs.

1. Unique expression patterns of lncRNAs: a sign of biological significance?

1.1: Are there circadian lncRNAs?

LncRNAs are fundamentally different from mRNAs in their capacity to encode a protein. At the same time, they share interesting similarities and differences to mRNAs in their structure, functions, and regulatory mechanisms. Almost all lncRNAs are transcribed by RNA Polymerase II (RNAP II) and modified with a 5'cap similar to mRNAs. However, only ~50% of lncRNAs have a poly(A) tail [13, 22, 23]. Nearly all lncRNAs are also spliced, although the number of exons are much fewer in lncRNAs [10, 13]. The expression level of lncRNAs is generally lower than that of mRNAs [10-14], however, this is not due to a difference in their RNA stability as the range of RNA half-lives is comparable between lncRNAs and mRNAs [24, 25]. Interestingly,

lncRNAs are more frequently localized in the nucleus than mRNAs [2, 13], suggesting that some lncRNAs play nuclear-specific roles without being transported into the cytoplasm for translation. Most remarkably, the expression patterns of lncRNAs are more specific in both space (i.e., tissue, cell type, and subcellular compartment) and time (i.e., developmental stage) compared to mRNAs, and very few lncRNAs are ubiquitously expressed [10, 12-14, 25, 26]. The high degree of specificity in lncRNAs' expression patterns have further bolstered the idea that some are functional.

The identification of rhythmically expressed mRNAs has been an active area of research over the past 20 years, as their protein products are believed to regulate output rhythms in biochemistry, physiology, and behavior. In mammals, ~5-40% of mRNAs in the entire transcriptome are rhythmically expressed depending on the tissue examined, and the rhythmicity of mRNA expression is highly tissue-specific [19, 27-30]. Similar to mRNAs, lncRNAs are also expressed rhythmically or differentially expressed between day and night in retina, liver, and pineal gland [18-21, 31]. Notably, the number of rhythmic lncRNAs is less than that of mRNA in a given tissue (Table 1). Meanwhile, the rhythmicity of lncRNA is even more tissue-specific compared to mRNAs, as fewer lncRNAs are commonly expressed rhythmically in multiple tissues [19, 20]. These data further support the idea that the spacio-temporal expression of lncRNAs is more tightly regulated than mRNAs.

Acute induction of mRNA expression by light also plays a prominent role in resetting the circadian clock. The light signal received by the retina activates the MAP kinase and cAMP signaling pathways in the suprachiasmatic nucleus (SCN) and induces mRNA expression of *Period 1* and *2* (*Per1, 2*) [32-35]. Interestingly, activation of the cAMP pathway also alters the

expression of several lncRNAs (both positively and negatively) in cultured pineal gland (Table 1) [21], suggesting that these lncRNAs contribute to the phase shift of the circadian clock.

It is still unclear whether these lncRNAs with unique expression patterns play any roles in regulating any parts of the circadian clock system: input, core or output [36, 37]. However, given that the metabolic cost of generating rhythmic transcripts is markedly higher than those of non-rhythmic transcripts [38], it is reasonable to assume that at least some of these lncRNAs are functional. If that is the case, how is the expression of lncRNAs regulated? What mechanisms ensure the unique expression patterns of lncRNAs, which are more tightly regulated than mRNAs?

1.2: How is the expression of lncRNA regulated?

Regulatory mechanisms of lncRNA transcription are considered very similar to that of mRNAs [13, 22, 23]. Indeed, the vast majority of lncRNAs (> 85 %) have a unique promoter [25, 39, 40] and contain conserved *cis*-regulatory elements [14, 22]. LncRNAs and mRNAs also appear to use the same epigenetic markers. Some histone modifications, such as H3K4me3, H3K9ac, and H3K27ac, are enriched in the promoter of actively transcribed RNAs, while others, such as H3K9me3 and H3K27me3, are enriched for transcriptionally inactive RNAs [12, 13, 22, 41, 42].

Rhythmic expression of mRNAs is generally driven by the core clock machinery, where core clock genes form interlocking transcription-translation feedback loops. At its core, BMAL1 and CLOCK (or its paralogue NPAS2) form a heterodimer and activate the rhythmic transcription of mRNAs with a *cis*-regulatory element, an E-box, such as *Period* (*Per*), and *Cryptochrome* (*Cry*). BMAL1/CLOCK also activates the expression of *Rev-erbα/β* and *Rora-c*,

whose gene products recognize REV-ERB/ROR binding motifs (ROREs) to repress or activate target mRNA expression, respectively. Additionally, BMAL1/CLOCK activates the expression of *Dbp,* while REV-ERB/ROR proteins regulate the expression of *Nfil3*, both of which recognize a D-box, and activate or repress target mRNA expression, respectively [43].

There are some indications that these transcription factors also drive rhythmic expression of lncRNAs. For example, ~20-30 % of REV-ERBα or BMAL1 recruitment is detected more than 10 kb away from any known mRNAs or ncRNAs, or sometimes even within an lncRNA itself [20, 44, 45], leading to the hypothesis that these recruitments are important for lncRNA expression. H3K4me3, H3K9ac, and H3K27ac modifications (i.e., markers for active transcription) also show robust rhythmic patterns near the transcription start site (TSS) of both mRNAs and lncRNAs, and their modification patterns coincide with the rhythmic recruitment pattern of RNAP II [18]. These data support the idea that regulatory mechanisms of rhythmic transcription are similar between lncRNAs and mRNAs.

Meanwhile, the regulatory mechanisms of rhythmic mRNA expression are still not fully understood. For example, BMAL1 recruitment is not sufficient for rhythmic mRNA expression, and the recruitment of both BMAL1/CLOCK and other transcription factors are required [46]. In addition, only ~20-40% of rhythmic mRNA expression is driven by *de novo* rhythmic RNA synthesis, suggesting that rhythmic RNA expression is regulated not only transcriptionally but also post-transcriptionally [47, 48]. Moreover, rhythmic histone modification in H3K4me3, H3K9ac, and H3K27ac, as well as RNAPII recruitment can be observed for non-rhythmic mRNAs, obscuring the significance of the rhythmic changes of these epigenetic markers in driving rhythmic mRNA [47].

Overall, even though the regulatory mechanisms of lncRNA transcription appear to be similar to that of mRNAs, how lncRNAs achieve highly specific expression patterns is poorly understood. Why is the rhythmicity of lncRNA expression more tissue-specific than mRNA? How do lncRNAs achieve higher tissue-specific rhythmic expression patterns if the mechanisms to drive rhythmic RNA expression is similar to mRNAs? These questions await further investigation.

2. Functions of lncRNAs：How can lncRNAs exert their functions without making a protein?

The advent of next-generation sequencing technologies has led to the discovery of a number of lncRNAs as discussed in the previous section [2, 10-14, 22, 25], however, the function of most remains unknown. This is mainly because of the lack of a systematic strategy in predicting the functions of lncRNAs based on their sequence or structure. Instead, empirical approaches, such as 'guilt by association,' can help infer function as well as potential interacting partners of lncRNAs based on expression patterns (i.e., concordant or discordant). Subcellular localization also provides a clue as to how lncRNAs function in a cell. Nuclear lncRNAs have a higher chance of interacting with nuclear architecture or chromatin to regulate chromatin remodeling and/or transcription. Cytoplasmic lncRNAs, on the other hand, can regulate translation, whereas other lncRNAs can reside in either cellular compartment and serve as precursors for small ncRNAs (i.e., miRNAs), or interact with RNAs and proteins important for a plethora of cellular physiology (Figure 2) [49-52].

Successful functional analyses have revealed that lncRNAs regulate almost all stages of gene expression, even though they do not encode a protein (Figure 2). There are two mechanistic

possibilities to achieve this: First is the transcript model, in which the transcripts of lncRNAs interact with DNA/chromatin, RNA, or protein to ultimately regulate target gene expression (Figure 2A). For example, transcripts of lncRNA can interact with the chromatin remodeling complex *in trans* and regulate the epigenetic markers of the target mRNA either positively or negatively. LncRNA transcripts can also facilitate chromatin loop formation to co-activate target gene expression that are distantly located, or directly interact with target RNA or a protein to alter their translation, RNA degradation, or protein localization [49, 52]. Second is the transcription model, in which the act of transcription, rather than the produced transcript, affects target gene expression in *cis* (Figure 2B). The transcription of lncRNA can affect the status of epigenetic marks of the target mRNA *in cis* either positively or negatively. Alternatively, a negative effect can be achieved by a mechanism called transcriptional interference where transcription in the opposite direction is interfered by multiple different mechanisms such as RNAP II collision, transcription factor displacement, nucleosome repositioning, and alteration of histone modifications [49, 52]. Below, we list a few examples of lncRNAs that target core clock genes and alter their cellular behavior. They use a wide array of regulatory mechanisms in different cellular compartments, providing important insights into the versatility of lncRNAs in exerting their functions.

2.1. NRON (Non-coding Repressor Of NFAT)

NRON was originally discovered to regulate nuclear localization of the NFAT protein in response to changes in intracellular $Ca2^{+}$ levels [53]. A recent study found that *NRON* also regulates the circadian clock via the NFAT-mediated calcium signaling pathway [54]. *NRON* interacts and co-localizes with PER2 at the perinuclear domain and inhibits phosphorylation of

PER2 as well as nuclear translocation of PER2/CRY [54]. In fact, *NRON* knockdown leads to longer period and reduced ~~rhythm~~ amplitude, consistent with the phenotype observed with an increased abundance of nuclear PER and CRY [54], providing mechanistic insights into how *NRON* regulates the circadian clock. Interestingly, *NRON* has two RNA regions that are almost perfectly conserved between primates and rodents [53], raising the possibility that functions of *NRON* in the circadian clock are conserved in these species.

2.2. Lnc-Crot

Lnc-Crot is a nuclear lncRNA that is rhythmically expressed in liver and kidney. *Lnc-Crot* forms a chromatin loop with other loci within the same chromosome and many circadian transcription factors are recruited to its loci. The loop formation *per se* is not rhythmic, however, *lnc-Crot* interacting loci have a slightly higher likelihood of being rhythmically expressed with the peak phase similar to that of *lnc-Crot* [20]. This led to the idea that *lnc-Crot* loci serve as a platform to co-regulate rhythmic expression of distantly located genes, even if they are not directly targeted by circadian transcription factors [20]. Because BMAL1 and REB-ERBβ also regulate rhythmic chromatin loop formation [27, 55, 56], it is possible that *lnc-Crot* and BMAL1 or REB-ERBβ work in concert to regulate target rhythmic gene expression.

2.3. HULC (Highly Upregulated in Liver Cancer) and *UCA1 (Urothelial Carcinoma-Associated 1)*

HULC is a cytoplasmic lncRNA originally discovered as the most upregulated transcript in hepatocellular carcinoma cells (HCCs) compared to non-neoplastic cells [57]. Coincidentally, *CLOCK* is also found to be upregulated in HCCs and this is because *HULC* interacts with the 5'

untranslated region (UTR) of *CLOCK* and stabilizes *CLOCK* mRNA [57, 58]. *UCA1* is another lncRNA that can be found both in the cytoplasm and the nucleus [59]. Both *UCA1* and *CLOCK* contain a miR-206 recognition motif and overexpression of miR-206 decreases the level of *UCA1* and CLOCK proteins. Interestingly, however, the repression of CLOCK by miR-206 can be rescued by the overexpression of *UCA1*, suggesting that *UCA1* serves as an miRNA sponge and prevents miR-206 from binding to *CLOCK* mRNA [59].

2.4. TCONS_00044595

TCONS_00044595 is rhythmically expressed in the pineal gland. The levels of *TCONS_00044595* and CLOCK are upregulated upon hypoxia; however, the increase of CLOCK upon hypoxia can be alleviated by knocking down *TCONS_00044595*. Because both *TCONS_00044595* and *Clock* are the direct target of miR-182, these data suggest that *TCONS_00044595* regulates CLOCK expression in an miR-182 dependent manner [60, 61].

2.5. FLRL2 (Fatty Liver Related LncRNA 2)

FLRL2 was originally discovered as one of the lncRNAs whose expression was down-regulated in mouse livers from a non-alcoholic fatty liver disease (NAFLD) model [62]. Knock-down or overexpression of *FLRL2* leads to a decreased or increased level of *Bmal1* mRNA, respectively, indicating that *Bmal1* is a downstream target of *FLRL2* [62]. The detailed mechanisms and functional relevance of *FLRL2* remain to be investigated.

The impact of some of these lncRNAs on the circadian clock itself (e.g., period, phase, or amplitude) is still unclear and remains to be investigated. Nevertheless, these examples clearly

showcase the diversity of lncRNAs in their mechanism of action (Table 2). Either the sequence or structure of lncRNAs are essential for their functions (i.e., transcript model) in most of the examples discussed above (Figure 2). However, the act of lncRNA transcription, not the lncRNA molecule itself, is the functional entity for another group of lncRNAs (i.e., transcription model) (Figure 2) [50, 51, 63]. In the next section, we focus on lncRNAs whose transcription, not transcript, is important for their functions and discuss how they regulate target gene expression and the circadian clock.

3. Evolutionary conservation of lncRNAs in the circadian clock system

The molecular architecture of core clock mechanisms is highly conserved from fungi to animals and consists of positive and negative regulators forming a negative feedback loop (Figure 3) [64-66]. Interestingly, natural antisense transcripts (RNAs transcribed from the opposite strand of a coding gene locus, Figure 1) for a negative regulator have been found in a few different species (Figure 3). The sense-antisense genomic arrangement intuitively suggests that they regulate each other and form an additional feedback loop. In addition, expression of the sense and antisense are rhythmic and antiphasic in some cases, whereas less than 10% of the genome-wide sense-antisense pairs have both transcripts expressed rhythmically with no obvious phase relationship between the two [18, 19]. These data strongly suggest that sense and antisense transcripts mutually inhibit each other and play an important role in the circadian clock.

How can a lncRNA and mRNA inhibit each other's expression? If the transcript model was employed, the sense and antisense transcripts would form an RNA duplex and decrease RNA stability (Figure 2). One limitation of this model could be that it requires both the sense and

antisense transcripts to be simultaneously present in the same compartment of the cell at the same time to maintain a stoichiometric balance, despite the expression level of antisense lncRNAs being generally much lower than sense mRNAs. Another transcript model could be that the antisense RNA interacts with the chromatin remodeling complex *in trans* and regulates the epigenetic markers of the sense mRNA. In contrast, if the transcription model was at play, transcriptional interference can be used to mutually inhibit each other's expression (Figure 2) [50-52]. We list some of these examples below and discuss how the act of transcription can regulate the core circadian clock machinery (Table 2).

3.1. Fungi

In *Neurospora*, the transcription factors WHITE COLAR (WC)-1 and -2 form a complex (WCC) that activates the transcription of *frequency* (*frq*). FRQ protein, in turn, represses WCC activity by inhibiting its interaction with DNA (Figure 3) [66]. *Frq* and its antisense lncRNA, *qrf* (backwards of *frq*), are both located on the same chromosome and completely overlap with each other (Figure 3) [67, 68]. Both *frq* and *qrf* show rhythmic and antiphasic expression patterns and they, indeed, mutually inhibit each other's expression [67, 68]. This mutual inhibition is required for robust circadian rhythmicity in the absence of light [68] and is achieved by transcriptional interference (i.e., transcription model), in which antisense transcription between *frq* and *qrf* leads to the collision of RNAP II and induces premature transcription termination of both transcripts [68]. *Qrf* also introduces DNA methylation at the *frq* promoter region and first activates, then represses *frq* expression post-transcriptionally (i.e., transcript model) [69]. Interestingly, the expression of both *frq* and *qrf* is acutely induced by light and mutant strains lacking *qrf* exhibits abnormalities in light-induced phase shifts [67, 68],

suggesting that *qrf* also plays a role in entrainment. However, its regulatory mechanisms remain elusive and await further investigation.

3.2. Insects

In silkmoth, *Antheraea pernyi*, the *period* gene has an antisense lncRNA even though the sense and antisense transcripts are located on different chromosomes and show antiphasic expression patterns [70]. The story is a little more complicated in *Drosophila,* where the temperature-dependent splicing of *period (per)* regulates the antisense protein-coding gene, called *Daywake (dyw)*, located downstream of *per* (Figure 3) [71]. Splicing of the last intron of *per* (termed *dpmi8*) is temperature sensitive and its efficiency increases in colder temperatures [71, 72]. This subsequently increases the level of *dyw* and ultimately suppresses daytime sleeping behavior, the "siesta", which is more commonly observed at higher temperatures [71]. Because there is a slight overlap between *per* and *dyw* at their 3'-ends near *dmpi8* (Figure 3), it is hypothesized that a thermosensitive spliceosome assembly at the *dmpi8* locus is the key to regulating *dyw* levels [71]. This is a unique example of the transcription model, in which the act of splicing accompanied by transcription is required for its function. Notably, no other sense-antisense pair has been reported to use splicing efficiency for its function thus far; understanding the detailed regulatory mechanisms will yield novel insights into how sense-antisense transcripts regulate their expression level and, ultimately, behavior.

3.3. Mammals

Mouse *Per2* has an antisense transcript, termed *Per2AS,* whose transcription starts in the intron 6 of *Per2* [18, 19, 47, 48] (Figure 3). Human *PER2* also has an antisense transcript

(*PER2AS*), but its transcription is divergent and *PER2* and *PER2AS* presumably share the same promoter [73]. The sense-antisense pairing is not unique to *Per2AS* in mammals, and m*Per1*, m*Per3,* m*Bmal1,* and h*PER3* are also reported have antisense transcripts [19, 69, 74]. Human *PER4* (also known as *PER3-pseudogene 1*) has also been identified as a pseudogene and is expressed ubiquitously at a low level [75].

Per2AS and *Per2* also show rhythmic and antiphasic expression patterns, at least in a few tissues; therefore, they were originally hypothesized to mutually inhibit each other [19, 47, 73, 76]. Indeed, the transcription of *Per2AS,* but not its transcript, inhibits *Per2*. However, *Per2* activates *Per2AS* [73], indicating that *Per2AS* and *Per2* form a single negative feedback loop, unlike *frq* and *qrf* that form a double negative feedback loop. Regardless, *Per2AS* regulates the amplitude of the circadian clock in fibroblasts and a mathematical study predicted that *Per2AS* would increase the robustness of the circadian system, similarly to *qrf* in *Neurospora* [73, 76]. Interestingly, *Per2AS* activates *Bmal1* in *trans* in a cell-type specific manner [73], and its function may be interdependent on *Rorc* [77]. Because the core clock structure is much more complicated in mammals than in fungi, it is plausible that *Per2AS* rewires the circadian clock network by not only regulating *Per2* but also by regulating other components to, ultimately, control the amplitude. In addition, the functions of *Per2AS* may be tissue specific as *Per2AS* is expressed in tissues such as liver, adrenal gland, fat pad, bladder, kidney, colon, duodenum, and large and small intestines, but not in the central nervous system and other peripheral tissues including heart, placenta, spleen, and thymus [73]. It will be of great future interest to decipher the functions of *Per2AS in vivo*.

These studies clearly demonstrate that the existence of the sense-antisense pair of a negative regulator is conserved in multiple species, whereas their genomic structure and functions appear to have diverged. This leads to the question as to whether the relationship between the sense and antisense transcripts is under selective pressure and, if so, why the functions have diverged. It is possible that the sense-antisense relationship arose from a convergent evolution, just like the molecular clock structure, and their functions were independently established. Functional analyses of antisense transcripts in other species will be sure to shed light on how the sense-antisense structure has been conserved in the core clock machinery.

4. Conclusion and Future Perspectives

LncRNAs have attracted significant interest from the scientific community in the past decade and remarkable progress has been made in discovering thousands of lncRNAs as well as deciphering their function in various biological processes. Circadian rhythm is no exception and hundreds of lncRNAs have now been shown to either be rhythmically expressed or regulate the behavior of the core clock genes and the core machinery (Table 1). These numbers, however, are most likely underestimated, as current technologies are not fully tailored to detect lncRNAs that are expressed at a low level, are tissue-specific, and are often times without poly(A) tails.

Given the pervasiveness of lncRNAs in the genome, functional analysis of lncRNAs is key to fully deciphering our genetic code. Nevertheless, this has been a challenging task, mainly because of the lack of predictive power and difficulty in designing experimental strategies to decipher the functions of lncRNAs without knowing the functional RNA domains or the

mechanisms of action [52, 78]. A classic approach to determine the function of a gene is to perturb its expression and observe consequent phenotypes. Unfortunately, however, gene knock-down or overexpression will not be effective unless the lncRNA transcript is required for its function (i.e., transcript model). CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa) can manipulate the endogenous gene expression and, therefore, are applicable for lncRNAs that use either the transcript or transcription model [79, 80], although they will not be useful for distinguishing the two models. CRISPR-based approaches to delete or insert the regulatory region is promising, yet this includes a risk of directly altering the DNA sequence of exons or *cis*-regulatory elements of nearby protein-coding genes [52, 78]. Therefore, a combination of different approaches will be necessary to fully characterize the mechanisms of action and the functions of lncRNAs.

Functional analyses of lncRNAs *in vivo* are even more challenging, as reverse-genetic approaches have not always been successful and have often challenged conclusions from earlier studies [17, 81]. Despite mounting evidence that supports the functionality of lncRNAs, knockout animals have not exhibited any overt phenotypes even for highly abundant lncRNAs whose functions are well-characterized in cell culture systems [17, 81]. This certainly fueled skepticism towards the functionality of lncRNAs and highlighted the difficulty in validating the functions of lncRNAs *in vivo*. It is still enigmatic as to why there is a striking phenotypic discord; one possible explanation is that initial characterizations overlooked subtle differences in the modes of action (i.e., transcript vs transcription models) and knockout animals were not designed appropriately to uncover the phenotypes. More targeted approaches that consider the regulatory mechanism of individual lncRNA will be crucial to designing knockout models and testing the functions of lncRNAs *in vivo*. Rescue studies will also serve as a crucial control

experiment to establish the modes of action and functions of lncRNAs in both cell culture and *in vivo* [17, 81].

Studies in the past decade have expanded the scope of lncRNAs from mere "junk" to functional molecules, at least for some. At the same time, the extent of how many lncRNAs out of the entire collection are functional while others are indeed junk is an ongoing and vigorous argument. The next big challenge in the field will be to assess whether additional lncRNAs are involved in the core clock machinery and to determine their functions, not only in mammals but in other organisms as well. Additionally, it will be of interest to gain insights into whether antisense transcripts for *Per1*, *Per3*, and *Bmal1* are important for regulating their sense mRNA expression and, ultimately, the core clock machinery. We have already developed many genetic and computational tools to address these questions and future studies will help uncover a more comprehensive view of how this new class of regulatory RNAs contribute to the circadian clock and other biological systems.

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Figure Legends:

Figure 1. Types of lncRNAs. LncRNAs are conventionally categorized by their location and orientation in relation to a nearby mRNA. Each box represents exons. Black: mRNA, red: lncRNA.

Figure 2. Regulatory mechanisms of lncRNAs. (A) Transcript model, in which the lncRNA itself (i.e., RNA molecule) is the functional unit. LncRNA transcripts interact with chromatin, other RNA, and protein, and regulate the behavior of target molecules. (B) Transcription model, in which the act of lncRNA transcription is the functional unit. LncRNA transcription alters histone modification patterns or collides with the transcription from the opposite strand.

Figure 3: Evolutionary conservation of the sense-antisense pairs in the negative limb and the core clock structure. (A) Genomic structure of sense-antisense transcripts. Arrows indicate the orientation of transcription. Each box represents exons. Black: mRNA, red: lncRNA. (B) Simplified core circadian clock structure consisting of transcription-translation feedback loops. Negative elements: orange, positive elements: green. FRQ: *frequency*, WC-1/2: *white collar-1/2*, PER: *period*, TIM: *timeless*, CLK: *clock*, CYC: *cycle*, PER1/2: *Period1/2*, CRY1/2: *Cryptochorome1/2*, CLOCK: *Clock*, BMAL1: *Brain and Muscle ARNT-like 1*.

Table 1: Identification of circadian-related lncRNAs

Tissue	Organism	Type	# Rhythmic lncRNAs	# Rhythmic mRNAs	Total detected genes	Rhythmicity Threshold	Ref
Liver	Mouse	Rhythmic	102	1160	11492 (coding & non-coding)	COSOPT p<0.02, Amp (FC)>2.0	18
12 Tissues	Mouse	Rhythmic	325 (32%)	43%	1016 mouse-human conserved lncRNAs	JTK_CYCLE, FDR<0.05	19
Liver	Rat	Rhythmic	604 clusters (7%)	2245	8705 lncRNA clusters	Yang et al., (25) FDR>0.17 (lncRNAs), > 0.20 (coding RNAs)	20
Liver	Rat	Rhythmic	790 clusters (11.8%)	NA	6713 lncRNA clusters	Cosine fitting p-value < 0.01	20
Pineal Gland	Rat	Difference between ZT7 and 17	112	NA	NA	FC>2.0 between ZT7 and ZT17	21
Pineal Gland (Culture)	Rat	cAMP pathway activation	8	NA	NA	NA	21
Retina	Mouse	Difference between ZT1.5 and 9	16 (intergenic)	365	13,296 (coding) 191 (intergenic)	FC>1.5 between ZT1.5 and 9	31

NA: Not applicable

Table 2: LncRNAs involved in the circadian rhythm regulation

LncRNA	Target gene	Organism	Regulatory Mechanisms	Effects on Circadian Rhythms	Ref
NRON	PER2 protein	Human U2OS cells	Transcript: Inhibition of PER2 phosphorylation and PER2/CRY nuclear localization	Longer period Reduced amplitude	54
Lnc-Crot	36 rhythmically expressed genes that interact with the *lnc-Crot* locus?	Mouse liver	Transcription?: Chromatin loop formation	Co-regulate target rhythmic mRNA expression?	20
HULC	*CLOCK* mRNA	Human hepatoma cells (L-O2 and HepG2)	Transcript: Complementary base pairing w/ 5'UTR	Unknown	58
UCA1	*CLOCK* mRNA	Human glioma cells (U251)	Transcript: miRNA sponge for miR-206	Unknown	59
TCONS_00044595	*Clock*	Mouse Pineal gland	Transcript: miR-182-dependent activation of CLOCK protein production	Unknown	60,61
FLR2	*Bmal1* mRNA	Mouse AML12 cells	Transcript: Unknown	Unknown	62
qrf	*frequency* mRNA	*Neurospora crassa*	Transcript: regulation of DNA methylation marks on the promoter; Transcription: transcriptional interference	Abnormalities in light-induced phase shifts and amplitude	67-69
Antisense of per	Unknown	*Antheraea pernyi*	Unknown	Unknown	70
Daywake (dyw)	*dpmi8* (*per* intron)	*Drosophila melanogaster*	Transcription (Splicing): a thermosensitive spliceosome assembly at the *dpmi8* locus regulated *dyw* levels?	Suppression of daytime "siesta" activity	71,72
Per2AS	*Per2* mRNA, *Bmal1* mRNA	Mouse fibroblasts	Transcription: regulation of *Per2* transcription via transcriptional interference, Transcript: regulation of *Bmal1* expression,	Changes in amplitude	73

Figure 1: Mosig and Kojima

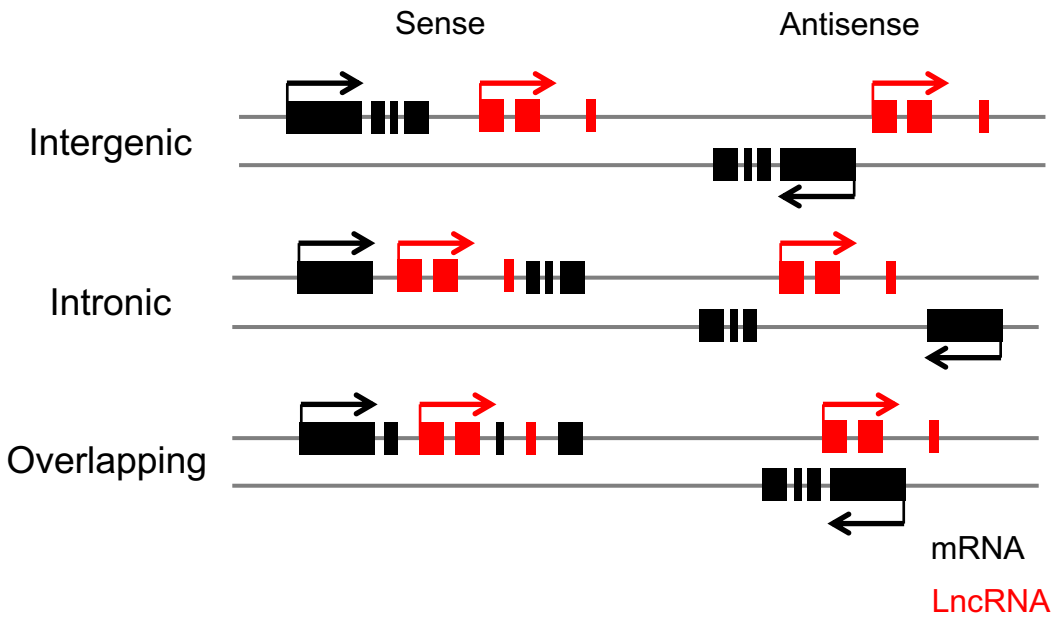

Figure 2: Mosig and Kojima

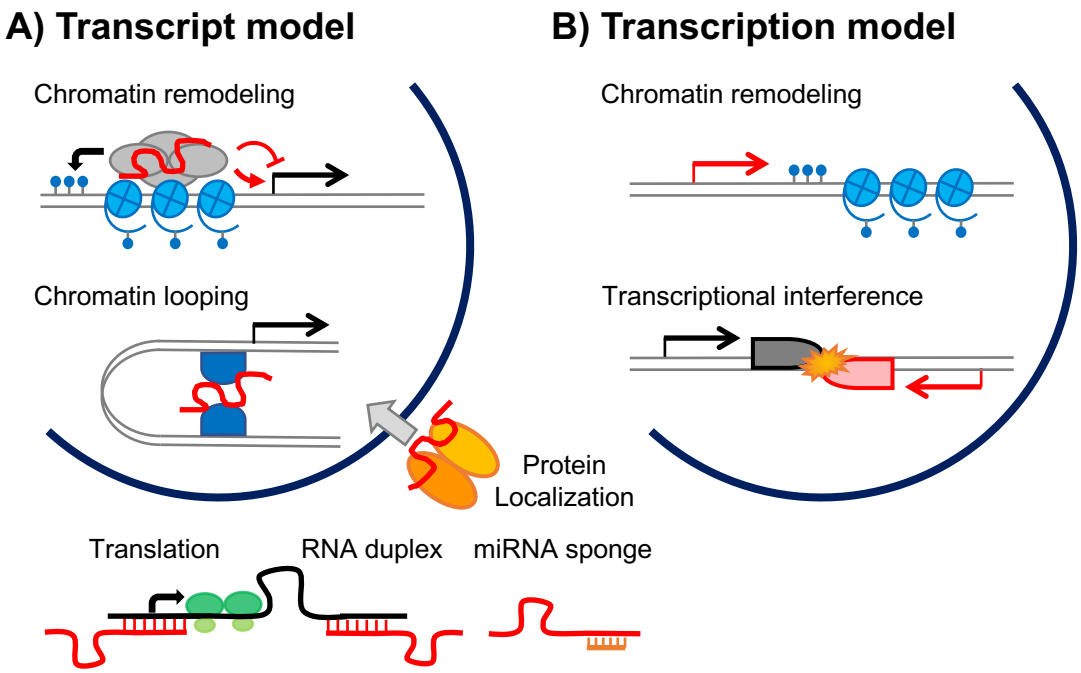

Figure 3: Mosig and Kojima