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Posttranscriptional controls - adding a new layer of control to clock gene expression

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Living organisms undergo biochemical, physiological and behavioural cycles with periods ranging from seconds to years. The cycles with intermediate periods rely on endogenous clocks that consist of oscillating gene expression. Our goal is to illustrate the modalities and specific functions of posttranscriptional controls of gene expression (exerted on pre-mRNAs and mRNAs) in biological clocks through two examples: the circadian clock and the vertebrate somitic segmentation clock, an embryonic clock with a period far below a day. We conclude that both uniformly and cyclically exerted posttranscriptional controls underpin the set-up of clock functions.

Rhythmic gene expression in oscillators

Living organisms are submitted to periodic oscillations of biochemical, physiological and behavioural parameters that are named biological rhythms. For a given process, the periods of the cycles range from less than one second to several years (Box 1). The biorhythms are subdivided into circadian (period approximately equal to 24 hours), ultradian and infradian (respectively shorter and longer periods, See Glossary) [1].

The present review will focus on essentially two rhythms, the ultradian rhythm that underpins vertebrate somitic segmentation and the circadian rhythm. During vertebrate embryo elongation, somites (presumptive muscles and bones) periodically bud off the non-segmented, posterior mesoderm (presomitic mesoderm). This results in a repetitive organization all along the antero-posterior axis, which is referred to as somitic segmentation. The periodic emergence of somites relies on an autonomous 'clock' within the non-segmented mesoderm that oscillates with a period ranging from 30 minutes in zebrafish to 2 hours in mice [2].

In circadian rhythms, there also exists an internal clock that is able to free-run with a period of approximately 24 hours. This clock exists in multicellular organisms, but also in yeasts [3]. This autonomous clock is temporally 'entrained' by light–dark or temperature cycles [4-6]. In mammals, it is located in the suprachiasmatic nucleus (SCN), a group of

hypothalamic neurons. Neuronal connections between the retina and the SCN explain the entrainment by light-dark cycles, which is evidenced among others by the resetting of the clock when light–dark cycles are shifted by some hours (in experimental conditions or following long-distance travels in humans) [4,5].

The mammalian circadian clock relies on eight proteins that are cyclically expressed in the SCN (Figure 1A): Clock [7], Bmal1 (Mop3) [8], Per1, Per2 and Per3 [9], Cry1 and Cry2 [10], and Rev-Erbα [11]. The Clock–Bmal1 complex controls the expression of several genes at the transcription level, among which *Period* (*Per1* to *Per3*), *Cryptochrome* (*Cry1* and *Cry2*), and *Rev-Erbα*, through its association with E-box elements. The Per–Cry protein complexes interact with and inhibit Clock–Bmal1, and Rev-Erbα inhibits the transcription of *Bmal1*. These two transcriptional feedback loops are responsible for the oscillations of Clock–Bmal1 activity that themselves account for the circadian expression of the clock outputs (Figure 1A) [4,5]. Several additional factors that modulate the mammalian circadian clock were recently identified by RNAi or proteomic approaches [12,13]. The circadian and the segmentation (Box 2) clocks both are set-up by transcriptional negative-feedback loops [2,14-18].

In addition to transcriptional loops, the control of the degradation of the proteins encoded by the clock genes determines their amounts in both clocks [17,19-21]. Several posttranslational modifications determine the activity and the stability of clock proteins [19]. Together, they represent a second layer of gene regulation in clock functions. A third layer of gene regulation must now be considered when investigating biological rhythms (Figure 1B). This layer, collectively referred to as posttranscriptional controls, encompasses all the regulations that are exerted at the RNA level (Box 3). They are mediated by ribonucleoproteic particles that include RNA-binding proteins (RNA-BPs) and non-coding RNAs, especially microRNAs (miRNAs) [22-24]. Their contributions in essential clock functions are an emerging and important field of study.

Circadian rhythms as a paradigm for dynamic posttranscriptional controls

The first evidence for posttranscriptional controls in circadian rhythms came from pioneering work in the fruitfly *Drosophila* [25]. Since, oscillating mRNA stability during the circadian cycle was also demonstrated in the mammalian core pacemaker (Figure 2). The stabilities of *Per2* and *Cry1* mRNAs vary during the cycle in mice, and, together with oscillating transcription, this results in rhythmic expression [26,27]. Woo and colleagues found that the RNA-BPs Ptbp1 and Hnrpd are able to bind to the 3' untranslated regions of *Per2* and *Cry1* mRNAs, respectively, and cause their rapid degradation [26,27]. Furthermore, the levels of cytoplasmic Ptbp1 and Hnrpd oscillate during the circadian clock and are correlated with target mRNA decay rates. In synchronized cultured cells, the oscillations of *Per2* and *Cry1* mRNAs were affected when the levels of Ptbp1 and Hrpd were reduced by RNAi. Together, these results suggest that oscillating amounts of cytoplasmic RNA-BPs may be responsible for the oscillating stability of target mRNAs that in turn determines their oscillating expression [26,27].

Rhythmic translation is another strategy to achieve cyclic expression of clock genes in the SCN, as demonstrated for *Per1* mRNA (Figure 2). The RNA-BP Rbm4 is cyclically expressed in-phase with Per1. It is able to bind to *Per1* mRNA and to stimulate its translation. Hence, translational stimulation by Rbm4 synergizes with transcriptional controls to amplify the level of Per1 oscillations [28]. Interestingly, only Rbm4 protein, but not *Rbm4* mRNA, is cyclically expressed, indicating that Rbm4 expression is itself controlled at a translational or posttranslational (protein degradation) level [28]. It is not known whether Rbm4 is required for circadian rhythms in whole mammalian organisms, but manipulating its level in cultured mammalian cells or in *Drosophila* affects circadian oscillations [28,29].

In addition to RNA-BPs, microRNAs (miRNAs) also control several mRNAs within the circadian pacemaker (Figure 2). miRNAs affect both mRNA stability and translation [22]. In animals, the interactions between miRNAs and target mRNAs are mediated by limited

sequence conservation. A miRNA can have several mRNA targets that are difficult to identify, although considering preferential evolutionary conservation improved the capacity to predict miRNA-mRNA interactions in silico [30]. Cheng and colleagues [31] showed that the miRNAs miR-219 and miR-132 have a circadian expression in the SCN, and they identified several potential mRNA targets. Per2 protein is overexpressed upon treatment with an antisense (antagomir) oligonucleotide against miR-132, which is consistent with miR-132 downregulating the translation of *Per2* mRNA. Furthermore, circadian period length and light-dependent clock resetting are altered in the absence of miR-219 and miR-132 respectively [31].

The SCN emits circadian signals to other regions of the brain, including the pineal gland. This gland synthesizes melatonin during the night and this circulating hormone relays the circadian rhythm to the peripheral organs. Arylalkylamine N-acetyltransferase (Aanat) is cyclically expressed in the pineal gland and is the rate-limiting enzyme in melatonin synthesis. Its expression is controlled at several levels, including mRNA stability and translation (Figure 2). The 3' untranslated region of *Aanat* mRNA contains a destabilizing element, and three rhythmically expressed RNA-BPs (Hnrnpr, Hnrnpl, Syncrip) are able to bind to this element and may play a role in the rhythmic degradation of *Aanat* mRNA [32]. In addition, *Aanat* mRNA is translated through an IRES (internal ribosome entry site), and Syncrip is able to bind to that IRES and stimulate *Aanat* mRNA translation. The oscillations of Syncrip protein during circadian cycles result in in-phase oscillations of *Aanat* mRNA translation, and manipulating the level of Syncrip impacts melatonin production in pinealocytes [33]. It is probable that the oscillations of Hnrnpr, Hnrnpl and Syncrip are themselves controlled by circadian cues sent by the SCN, but how this is achieved is unknown (Figure 2).

In addition to brain, most mammalian organs contain autonomous clocks that are entrained by cues emitted by the master clock [34], and posttranscriptional controls might operate in these peripheral clocks too. A comprehensive microarray experiment revealed

ultradian rhythmic expression of several genes in mouse liver [35]. This might indicate some ultradian clock, but an alternative cause could be mRNA degradation. If genes are transcribed following circadian rhythms and the corresponding mRNAs are degraded following a circadian, out-of-phase, rhythm, the mRNA levels might oscillate with a period of 12 hours [35].

A function for oscillating mRNA stability in circadian rhythm has also been described in plants. A microarray screening in *Arabidopsis thaliana* identified two mRNAs whose stabilities oscillate with a period of 24 hours. Disruption of the pathway responsible for the rapid degradation of these mRNAs in the afternoon alters the oscillations of these mRNAs in correlation with an altered circadian rhythm at the whole-plant level, indicating a link between circadian rhythms in plants and specific mRNA decay [36].

Clues for the importance of posttranscriptional controls in biological rhythms

How widespread are posttranscriptional controls of gene expression in biological rhythms? A rough estimate is provided by identifying factors that control gene expression at the posttranscriptional level and that display a rhythmic circadian expression. This is the case for several miRNAs in the plant *Arabidopsis thaliana* [37], fly heads [38] and mouse retinas [39].

Several examples of oscillating RNA-BPs have also been reported, in addition to the factors described in the previous section. In the green alga *Chlamydomonas reinhardtii*, the capacity of the RNA-binding complex CHLAMY1 to bind to target mRNAs follows a circadian rhythm [40]. CHLAMY1 comprises two subunits that both are RNA-BPs. Experimentally manipulating the level of either of these two subunits strongly interferes with the circadian rhythm, suggesting that these two proteins are at the heart of the circadian clock in this species [41]. The *Chlamydomonas* clock is entrained by temperature cycles, and both subunits of CHLAMY1 are involved in temperature integration [42]. In rats, the RNA-BP Mbnl2 (Muscleblind 2) that is involved in alternative splicing of pre-mRNA has an oscillatory

153 expression in the pineal gland [43]. Finally, Nocturnin, a poly(A) ribonuclease (that causes
154 mRNA decay and translational repression by removing the poly(A) tails, see Box 3), is
155 cyclically expressed in the retina [44]. Surprisingly, mice in which the *Nocturnin* gene has
156 been inactivated display normal circadian rhythms and expression of clock genes (but altered
157 lipid metabolism or uptake) [45]. Hence, factors that control mRNA fate and display a
158 rhythmic expression pattern can be divided into two groups: those that directly influence the
159 clock, and those, like Nocturnin, that represent its readouts.

160 An additional clue to estimate the extent of translational controls in biological rhythms
161 is to compare the levels of cycling proteins with their corresponding mRNAs. Systematic
162 comparison of the transcriptome and the proteome of mouse liver showed that only half of the
163 genes that exhibit rhythmic protein expression also exhibit rhythmic mRNA expression [46].
164 Interestingly, circadian variations in protein isoforms were also reported by these authors,
165 which are consistent with circadian modifications of alternative splicing [46]. The strong
166 discrepancies between transcriptome and proteome data suggest prevalent translational and/or
167 posttranslational (protein degradation) controls of cyclic gene expression in the circadian
168 clock.

169

170 **One step forward: how are cyclic posttranscriptional controls generated?**

171 As seen above, cyclical posttranscriptional controls are exerted on several mRNAs and
172 in several physiological systems. In some already discussed cases, the factors involved in
173 RNA regulations are uniformly expressed, but their activity or subcellular localisations
174 oscillate [26,27,41]. The mechanisms underlying these oscillations are unknown.

175 The factors controlling mRNA fate may also themselves be cyclically expressed,
176 owing to a cyclical transcriptional regulation, as demonstrated for miR-219 (see Figure 2)
177 [31], but also owing to posttranscriptional negative-feedback loops. In *Neurospora crassa*,
178 FRQ and FRH proteins form the FFC complex, which is able to recruit the RNA exosome (a
179 multi-subunit complex involved in mRNA degradation [47]) to *frq* mRNA, and to thereby

cause its degradation. Together with the capacity of FFC to repress the transcription of *frq* gene, this posttranscriptional negative-feedback loop achieves circadian oscillations in *N. crassa* [48]. In *Arabidopsis thaliana*, *At*GRP7 and *At*GRP8 are two RNA-BPs with a circadian expression. *At*GRP7 overexpression ablates circadian expression of *Atgrp7* and *Atgrp8* mRNAs [49]. Both proteins are able to bind to their own pre-mRNAs and direct their splicing pathways towards mRNA isoforms that contain a premature termination codon. These isoforms are rapidly degraded by the non-sense-mediated mRNA decay (NMD) pathway (see Box 3). Consequently, *At*GRP7 and *At*GRP8 negatively auto-regulate and cross-regulate their synthesis [50,51]. This mechanism very probably ensures a cyclical stability of the mRNAs encoding *At*GRP7 and *At*GRP8, which contributes to their circadian oscillations.

In mammals, the RNA-BPs Rbm4 and Syncrip display oscillating expressions [28,33]. It is tempting to speculate that these oscillations result from negative auto-regulations similar to plant *At*GRP7 and *At*GRP8 or N. crassa FRQ. Indeed, several mammalian RNA-BPs negatively regulate their own synthesis. PTBP1 and PTBP2 regulate the splicing of their own respective pre-mRNAs and promote the skipping of an exon that results in an NMD sensitive transcript [52,53]. They also cross-regulate each other through this splicing event [54,55]. Similarly, the RNA-BP Celf2 negatively autoregulates its synthesis by inhibiting the splicing of its own pre-mRNA [56]. Whether these negative auto-regulations of RNA-BPs generate oscillations, and how these putative posttranscriptional negative-feedback loops are interconnected with the master transcriptional loop, have not been tested in mammals.

Posttranscriptional controls do not need to be cyclically exerted to play a role in biological rhythms.

Transcriptional negative-feedback loops result in successive activations and repressions of gene promoters. When transcription is shut off, mRNAs decay following exponential kinetics. If the decay of a given mRNA is sufficiently rapid (short half-life) relative to the period of transcriptional oscillations, then almost complete removal of the

mRNA will occur before transcription resumes. This situation produces oscillations of mRNA of maximum amplitude. However, if the transcription resumes before the mRNA is completely degraded, then the amplitudes of the mRNA oscillations are reduced or the oscillations are damped and, at the extreme of very stable mRNAs, completely disappear. Therefore, rapid mRNA degradation is required to convert switches between active and inactive transcription into oscillatory amounts of the corresponding mRNAs. One could predict therefore that rapid and uniform mRNA decay is instrumental in the generation of short-period (ultradian) biorhythms, and this prediction has at least been partially confirmed in the case of vertebrate somitic segmentation clock.

The period of the somitic segmentation clock is comprised between 30 minutes and 2 hours [2]. Within one period, the amounts of several tens of mRNAs oscillate [57]. It takes no more than a few minutes to have a cyclic mRNA completely degraded, indicating very short half-lives. The data demonstrating the occurrence of posttranscriptional controls in somitic segmentation are summarized in Table 1.

The expression pattern of *Lunatic Fringe* (*Lfng*, a modulator of Notch signalling, one of the pathways required for segmentation) has been described in mice. In situ hybridizations were made with both an exonic probe to reveal the mRNA and an intronic probe to reveal sites of active transcription. The staining patterns with these two probes were very similar, demonstrating that *Lfng* mRNA is degraded virtually as rapidly as the *Lfng* introns [58]. Since splicing occurs co-transcriptionally, and excised introns are very rapidly degraded, these data demonstrate the remarkable instability of *Lfng* mRNA.

Reporter genes also showed that mRNA degradation is required to achieve the dynamic expression pattern of the clock genes. In Zebrafish, a GFP reporter controlled by the *Her1* promoter (an oscillating component of the core clock) accumulates in the presomitic mesoderm owing to its high stability, suggesting *a contrario* the rapid decay of the endogenous mRNA [59]. In *Xenopus* transgenic embryos, a characteristic striped expression pattern of *Hairy2a* and *Bowline,* two genes downstream of the clock, is recapitulated by

234 reporter mRNAs only if they contain a destabilizing element in their 3' untranslated regions
235 (3'UTR) [60,61]. Taking as evidence for rapid mRNA degradation the capacity of a 3'UTR to
236 confer upon a reporter GFP gene a striped pattern of expression, several chick or mouse clock
237 mRNAs can be considered as unstable (Table 1 [60]). More recently, an approach combining
238 *in ovo* electroporation and an inducible promoter showed that chick *Lfng* mRNA is
239 destabilized by means of its 3'UTR [62].

240 What happens to segmentation if the rapid degradation of the cyclic mRNAs is
241 impaired? Computational models of the zebrafish segmentation clock predict that the
242 oscillations of the core clock genes are sustained only if the corresponding mRNA and
243 proteins are unstable [18,63], but this was not experimentally tested at the mRNA level. In
244 Zebrafish, the '*tortuga'* mutant shows an altered pattern of expression of *Her1* with impaired
245 oscillations that is consistent with mRNA stabilisation [64]. The corresponding wild-type
246 gene product may therefore be responsible for the rapid decay of *Her1* mRNA. This gene has
247 not been identified. In *Xenopus*, the RNA-BPs Celf1 and Fxr1p regulate the stability and/or
248 the translation of bound mRNAs, and knock-down of these proteins causes segmentation
249 defects [65,66]. This suggests that these proteins have to bind and control a subset of mRNAs
250 for correct segmentation to occur. The mRNA encoding Su(H), that is involved in Notch
251 signalling in the segmentation clock, was identified as a target of Celf1. Specifically, a
252 functional interaction between Celf1 and *Su(H)* mRNA is required for both the degradation of
253 this mRNA and somitic segmentation [67]. Together, these data show that uniform mRNA
254 regulation plays a key role in oscillations of the segmentation clock.

255 Continuous posttranscriptional controls were also described in the circadian clock. The
256 expression of the microRNAs miR-192 and mi-R194 in cultured mammalian cells [68], miR-
257 122 in mouse liver [69] or *bantam* in fly heads [70] apparently does not follow a circadian
258 cycle (although miR-122 is cyclically transcribed but remains at approximately constant
259 levels due to a long high-life [69]). All these miRNAs continuously downregulate identified
260 target mRNAs encoding proteins involved in the circadian clock, and manipulating their

levels modifies the period and/or amplitude of circadian oscillations [68-70]. Other examples are given by *Per1* and *Per3* mRNAs that are uniformly unstable in NIH3T3 cells and transgenic mice, respectively [71,72]. The circadian oscillations of *Per3* mRNA are strongly modified when its mRNA degradation element is deleted [71]. Hence, constant posttranscriptional repression may be required in some instances to achieve optimal circadian oscillations in addition to cyclical posttranscriptional controls of gene expression.

Concluding remarks and future directions

The comparison of the segmentation and circadian clocks paves the way for future researches (Box 4). Both mRNA degradation and translation, mediated by RNA-BPs and miRNAs, have recognized functions in the circadian clock. In several instances, translational efficiency and mRNA degradation oscillate in the circadian clock, and these oscillations fully contribute to the clock. By contrast, the only known mode of posttranscriptional control in the segmentation clock is constant mRNA degradation. In fact, we might simply lack data concerning the different modes of posttranscriptional controls in the segmentation clock. Using the circadian clock as a paradigm for posttranscriptional controls in clocks, we recommend that the various modes of oscillating posttranscriptional controls should be carefully investigated in the segmentation clock. Furthermore, most but not all known modes of posttranscriptional controls were described in the circadian clock. Specifically, we know nothing about the subcellular localization and the putative localized translation of the mRNAs encoding factors of the clock. It might be of interest to investigate these points in the regulation of mammalian circadian clock considering their recognized importance in neurons [73].

Another question is whether there exist human diseases caused by posttranscriptional defects in clocks. Congenital vertebral malformations are often of genetic origin. Some of them were associated with mutations affecting genes of the segmentation clock, but the aetiology of most of them is unknown [74]. Factors involved in posttranscriptional regulations

in somitic segmentation, most of which were not identified, will be potential candidates for causing these syndromes. Also several human troubles arise from defects in the circadian clock, such as sleep disorders. Interestingly, fragile X patients suffer from sleep disorders [75]. This syndrome is a consequence of impaired expression of the RNA-BP FMR1, and *Fmr1* KO mice display an altered circadian rhythm [76]. Fragile X syndrome provides therefore a link between posttranscriptional controls, human pathology and the circadian clock, and it can be anticipated that this will not remain an isolated example.

A last issue is the extent of posttranscriptional controls in clocks. Several inactivations of gene encoding RNA-BPs were reported in mice. Some of them may be at the origin of circadian troubles that remained unnoticed up to now, and this would merit careful reinvestigation. For the RNA-BPs whose inactivations lead to clock troubles, the arising question will be the identity of the mRNAs that are normally associated with that protein and are deregulated upon its inactivation (and whose deregulation is responsible of the observed troubles). Recent technological breakthroughs allow some optimism concerning our capacity to ask that question. "CLIP" (Cross-linking and immunoprecipitation) allows the co-immunoprecipitation of RNA-BPs and associated RNAs [77]. Combined with next-generation sequencing, it permits the genome-wide identification of the RNAs bound by a protein ('CLIPseq') [78-80]. Maps of the interactions between miRNAs and mRNAs were drawn from Argonaute CLIPseq [81,82]. Together, these recent technologies will provide us with a genome-wide characterization of the network of posttranscriptional controls in virtually any cell type, including those subject to clock oscillations, and will allow us fully appreciating the extent of posttranscriptional controls in clocks.

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Glossary box

3' Untranslated Region (3'UTR): Region of the mRNA 3' to the translation stop codon.

Alternative splicing: Various ways to skip introns and splice exons. This mechanism generates a large diversity of mRNA molecules from a single gene. Alternative splicing includes mutually exclusive exons (where splicing leads to the inclusion of either of two exons), exon skipping, intron retention, alternative 5' or 3' splice sites (leading to the retention of all or only part of an exon) and alternative terminal exons.

Circadian rhythm: A cycle one day long (Latin *circa*, about, and *dies*, day). The period of a circadian rhythm is 24 h when the organism is grown under a light-dark cycle (12h light, 12h darkness), and about 24 h when the organism is released into free-running condition. Several parameters cycle in circadian rhythms, the most obvious one in mammals being sleep and wake.

Free-running rhythm: Circadian rhythm in the absence of external cues (like constant darkness and temperature).

Half-life. Time required in the absence of synthesis to achieve degradation of half the initial amount of a molecule (like an mRNA).

I**nfradian rhythm:** A cycle of length above 24h.

Melatonin: Circulating hormone secreted by the pineal gland during the night in mammals. It relays the circadian rhythm imposed by the central nervous system to the peripheral organs.

miRNA (micro RNA): Short double-stranded RNA, encoded by the genome, that controls gene expression at several levels. In vertebrates, a prevalent feature of miRNAs is their capacity to specifically repress the translation of target mRNAs by (limited) sequence complementarity.

Period: Time interval between two reference points (two peaks for example). Inverse of frequency.

Presomitic mesoderm: Posterior, non-segmented mesoderm, in which the segmentation clock is active and from which segmented somites periodically bud off.

Somites: Transient embryonic repeated mesodermal structures. They are the origin of adult skeletal muscles, bones and derm.

Somitic segmentation: Organisation of the somites as repeated units along the embryonic antero-posterior axis.

Suprachiasmatic nucleus (SCN): A region of the hypothalamus. The master circadian clock is located within the SCN.

Ultradian rhythm: A cycle of length shorter than 24h (e.g. the segmentation clock).

Box 1. Some examples of biological rhythms

Depending on the period, biorhythms are classified as ultradian (period T<24h), infradian (T>24h) and circadian (T~24h). Ultradian rhythms include heart beating (T=fractions of seconds to seconds), sleep episodes (T=tens of minutes), respiratory oscillations in yeasts (T=1–5h [83]), somitic segmentation in vertebrates (T=30 minutes in Zebrafish, 2h in mice [2]), or pulses of LH secretion by the pituitary gland (T~3h in men [84]). Infradian rhythms include successions of torpor and arousal during the hibernation of small mammals (T=several days [85]), female menstrual cycles (T=several days to several months), annual rhythms (flowering of most plants), and even pluri-annual rhythms such as the emergence of Cicada [86].

Box 2. The vertebrate segmentation clock.

Please refer to the accompanying figure.

Title of the figure "The zebrafish core segmentation clock"

In zebrafish, the core segmentation clock consists of Her1 and Her7 proteins (see Figure). Homodimers or heterodimers of these proteins bind to their own promoters and repress their transcription. Taking into account transcriptional and translational delays, this results in oscillating levels of these proteins. Furthermore, Her1/7 duplexes repress the transcription of Delta-C, a transmembrane Notch ligand. When bound by its ligand, the Notch transmembrane receptor undergoes a limited proteolysis that releases the Notch intracellular domain (NICD) in the cytoplasm. NICD is then translocated to the nucleus. Together with Su(H) protein, the NICD stimulates the transcription of target genes including *Her1* and *Her7*. The stimulation of *Her1/7* transcription by Delta-C expressed in adjacent cells, and the ensuing repression of *Delta-C* gene by Her1/7 achieves coordinated oscillations in neighbouring cells [18,63]. Her13.2 reinforces the transcriptional inhibition mediated by Her1/7, and it is controlled by the FGF pathway. This links the Notch and FGF signalling pathways [87]. Several other genes are downstream of Her1/7 and are involved in somitic segmentation. In amniotes (chick, mouse), the segmentation clock is more complex. It requires oscillations of the Notch modulator Lunatic fringe, and of tens of mRNAs that encode proteins belonging to the FGF and Wnt signalling pathways in addition to Notch [57].

Box 3. Different levels of posttranscriptional controls of gene expression.

Please refer to the accompanying figure.

Title of the figure "pre-mRNA and mRNA fate in eukaryotic cells"

The posttranscriptional controls are exerted on RNA molecules and are indicated in red on the figure. Concomitantly with nuclear transcription, pre-mRNAs are matured to mRNAs. Pre-mRNA maturation refers to three events: 5' capping, 3' cleavage and polyadenylation, and intron excision coupled with exon splicing. Most pre-mRNAs can be cleaved and polyadenylated at several sites (alternative cleavage/polyadenylation) and/or undergo several splicing patterns (alternative splicing. In the figure, the second exon is either skipped or spliced). Due to alternative cleavage/polyadenylation and alternative splicing, a large variety of mRNAs can be obtained from a given pre-mRNA.

After nucleo-cytoplasmic export, mRNA translation and decay are controlled, and the 3' poly(A) tail is a major site for these controls. Polyadenylated mRNAs are much more actively translated than deadenylated mRNAs. The initiation factor eIF4G, that recruits the small ribosomal subunit, is able to interact simultaneously with the 5' cap-binding protein eIF4E and the 3' Poly(A) binding protein. The connection between mRNA 5' (cap) and 3' (poly(A) tail) ends strongly stimulates translation [88]. In addition, polyadenylated mRNAs are much more stable than deadenylated mRNAs. For most mRNAs, deadenylation is the rate-limiting step of mRNA decay, and several factors that control mRNA stability do so by regulating the deadenylation rate. In higher eukaryotes, the major pathway for mRNA decay is poly(A) tail removal (deadenylation) followed by RNA exosome-mediated 3' to 5' exonucleolytic degradation. [89]. The 5'-most AUG codon is generally the translation initiation codon, but more distal initiation codons can also be used (alternative initiation of translation), resulting in alternative protein isoforms. This mechanism was described for instance for the mRNA that encodes FRQ, a component of the *N. Crassa* circadian clock [90].

Nuclear and cytoplasmic controls are tightly coupled. A complex (EJC, exon junction complex) is assembled during splicing immediately upstream of exon junctions, and remains associated with the mRNA during nucleocytoplasmic export. This hallmark of a nuclear event then influences cytoplasmic mRNA translation and degradation [91]. For example, the EJC is involved in the recognition and rapid degradation of mRNAs containing a premature stop codon by the 'nonsense-mediated mRNA decay' (NMD) pathway [91]. In addition, alternative splicing can lead to mature transcripts that contain alternative 3' untranslated regions (3'UTR), that are instrumental in mRNA stability and translation [88]. Consequently, alternative cleavage/polyadenylation or splicing impacts mRNA half-life or translation.

Box 4. Future questions

- Uniform mRNA instability is the only mode of posttranscriptional controls demonstrated in the segmentation clock. Do oscillating mRNA stability and/or oscillating mRNA translation also play a role?

- In the circadian clock, the described mechanisms relate to most posttranscriptional controls found to be governing the expression of other non-clock-related gene programs, but mRNA intracellular traffic and local translation were not reported. Since they are prevalent mechanisms in neurons [73], one could ask if they have a function in the circadian clock.

- A posttranscriptional feedback loop was demonstrated in *N. crassa* circadian clock [48], and the levels of some RNA-BPs oscillate in mammalian circadian clocks [28,33]. Are there posttranscriptional feedback loops in vertebrate clocks that could account for the oscillations of these RNA-BPs?

- Systematic gene inactivations were reported in lower metazoans [92,93], and several genes were disrupted by homologous recombination in mice. Some of them encode RNA-BPs or miRNAs. Which inactivations lead to clock troubles, demonstrating an involvement of the corresponding gene products in clock setting or robustness?

- What are the posttranscriptional networks in clocks? For the RNA-BPs and the miRNAs that are involved in clocks, what are the associated mRNAs?

- Are deregulations of posttranscriptional networks in clocks at the origin of human diseases?

Figure legends

Figure 1. The mammalian circadian clock and its three layers of control

(a) Master circadian pacemaker in the suprachiasmatic nucleus (SCN). The Clock–Bmal1 complex directly stimulates the transcription of *Per*, *Cry, Rev-Erbα*, and of output clock-controlled genes (CCGs) via binding to the E-box. Oscillatory activity of the Clock–Bmal1 complex is achieved by two negative feedback loops: the Per–Cry complex inhibits Clock–Bmal1, and *Bmal1* transcription is repressed by binding of Rev-Erbα to the RRE (ROR response element). **(b)** Relationships between transcriptional, posttranscriptional and posttranslational layers in the control of *Per* genes expression. Since Per proteins contribute to the control of the Clock–Bmal1 complex, fine-tuning their levels is required to obtain oscillations of clock genes. The levels of Per proteins are regulated at a transcriptional level (yellow layer) by the Clock–Bmal1 complex (see Figure 1a). They are regulated at a posttranslational level too (green layer), among others as Casein-kinase1-δ and -ε mediate Per phosphorylation that targets them to ubiquitin/proteasome degradation [19,20]. Recent results demonstrate that a third layer (posttranscriptional controls, red) should be added to complete the picture. The oscillating controls (transcription, mRNA translation and degradation) are in capital letters.

Figure 2. Posttranscriptional controls exerted on mRNAs encoding proteins involved in circadian rhythms

Arrows and blunt-end lines towards ribosomes (brown) indicate stimulation and inhibition, respectively, of mRNA translation. Arrows towards the exonucleolytic enzyme (yellow) indicate stimulation of mRNA decay. The sinusoidal symbols on the right of the factors involved in posttranscriptional controls indicate oscillating levels of these factors. **(a)** Components of the master circadian clock in the SCN. **(b)** Aanat, a pineal, rate-limiting enzyme in melatonin synthesis.

Gene	Function in the clock	Evidence for posttranscriptional controls	References
Llnfg in amniotes	Encodes modulator of Notch signalling	mRNA instability inferred from expression pattern in mice; 3'UTR of chick mRNA confers rapid degradation to a reporter mRNA	[58,62]
zebrafish *Her1*	Encodes component of the core clock	The expression pattern of a reporter mRNA controlled by *Her1* promoter is different from that of endogenous *Her1* due to increased mRNA stability. Expression pattern in the *Tortuga* mutant consistent with *Tortuga* gene product being responsible for *Her1* mRNA instability	[59,64]
Xenopus Hairy 2a, *Hairy 1*, *Esr5*, *Nrarp*, *Bowline*, Chick *Hairy 1*, Mouse *Hes1*, human *HES4*	Mouse Hes1 and human HES4 may be components of segmentation clock. The other genes encode factors downstream of the segmentation clock. Some of them are involved in setting the antero-posterior polarity of forming somites	In *Xenopus*, the 3'UTR of *Hairy 2a* confers instability on a reporter mRNA. The expression pattern of *Hairy 2a* or *Bowline* was recapitulated in transgenic embryos with the appropriate promoter and a 3'UTR of one of these genes, but not with a 3'UTR of a stable mRNA.	[60,61]
Xenopus Su(H) (homologue of mammalian *Rbpj*)	Binds to Notch intracellular domain to stimulate expression of Notch target genes	mRNA instability is conferred by association with the RNA-BP Celf1. A specific impairment of the interaction between Celf1 and *Su(H)* mRNA causes segmentation defects.	[65,67]

674

675 **Table 1. Posttranscriptional controls of gene expression in the segmentation clock.**

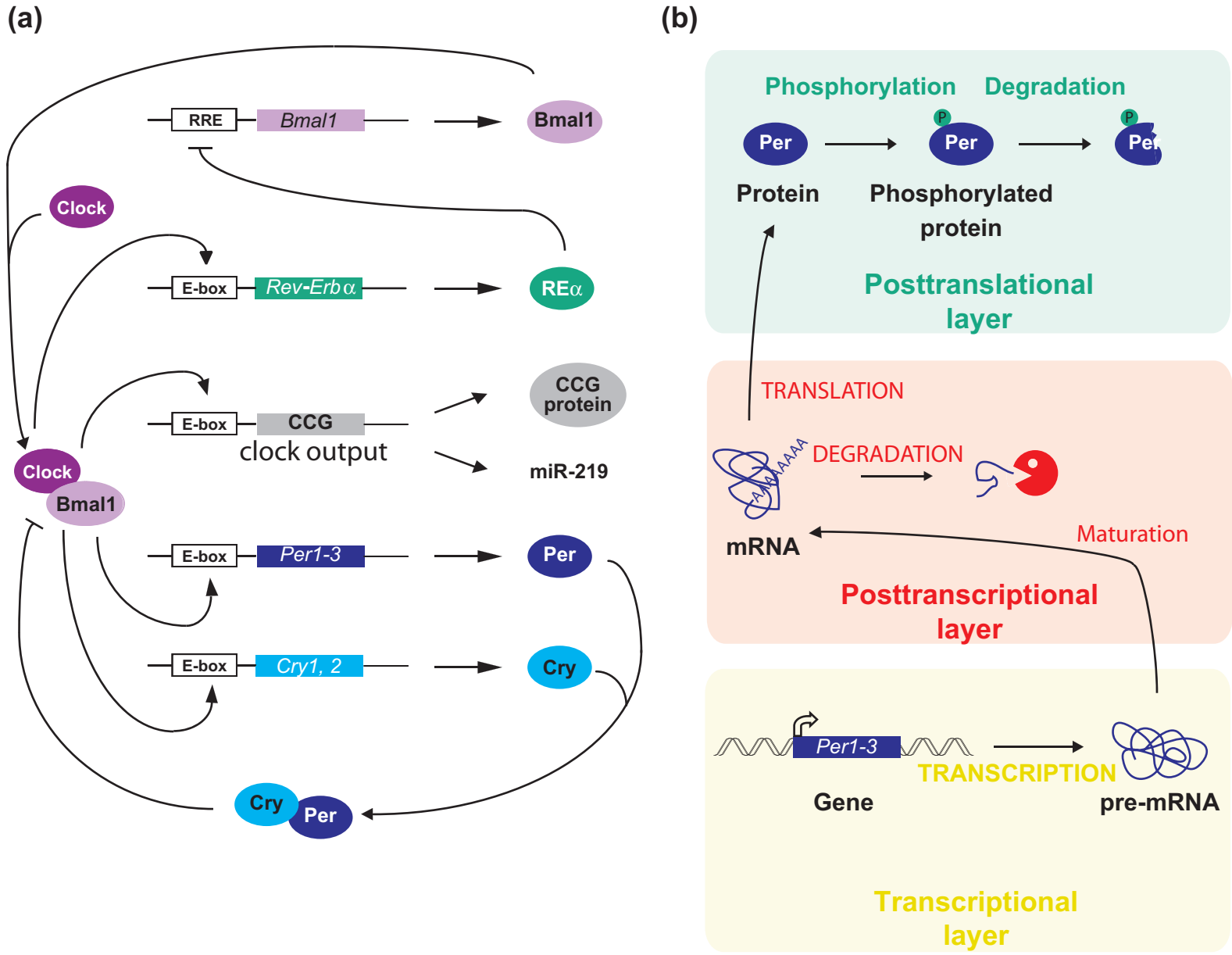
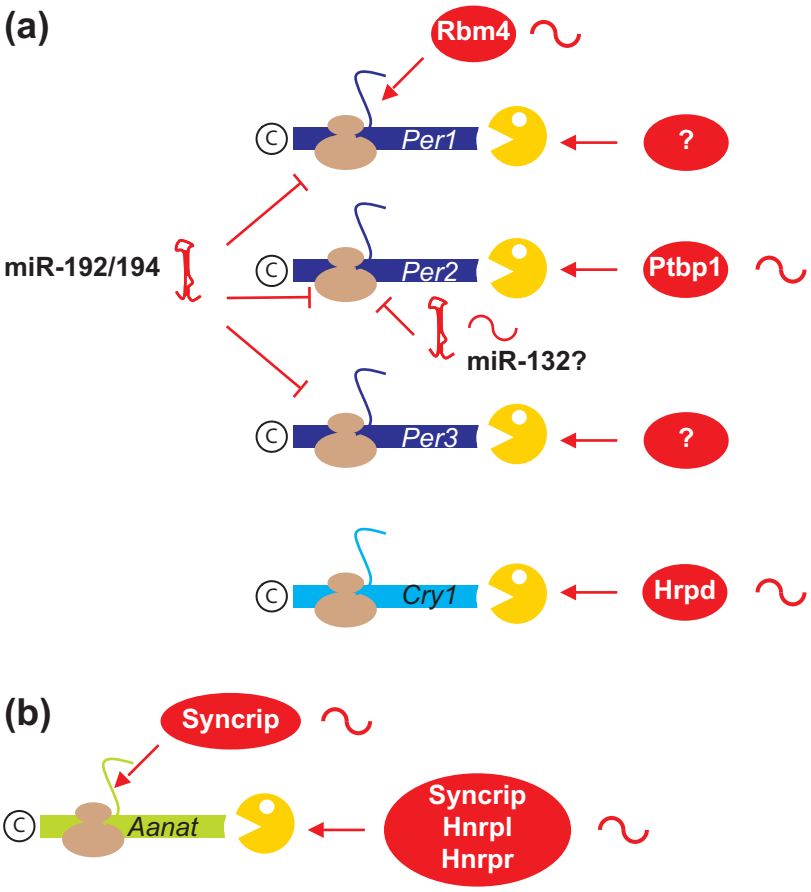

(a)
RRE
Bmal1
Bmal1
Clock
E-box
Rev-Erbα
REα
E-box
CCG
clock output
CCG protein
miR-219
Clock
Bmal1
E-box
Per1-3
Per
E-box
Cry1, 2
Cry
Cry
Per
(b)
Phosphorylation
Degradation
Per
Per
P
Per
P
Protein
Phosphorylated protein
Posttranslational layer
TRANSLATION
DEGRADATION
AAAAAAA
mRNA
Maturation
Posttranscriptional layer
Per1-3
TRANSCRIPTION
Gene
pre-mRNA
Transcriptional layer

FIGURE 1


(a)
Rbm4
Per1
?
miR-192/194
Per2
Ptbp1
miR-132?
Per3
?
Cry1
Hrpd

(b)
Syncrip
Aanat
Syncrip
Hnrpl
Hnrpr

FIGURE 2

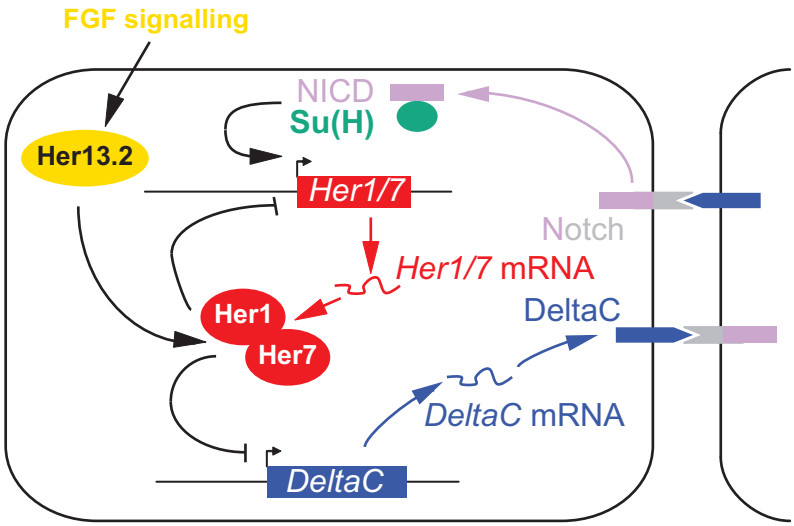


FIGURE OF BOX 2

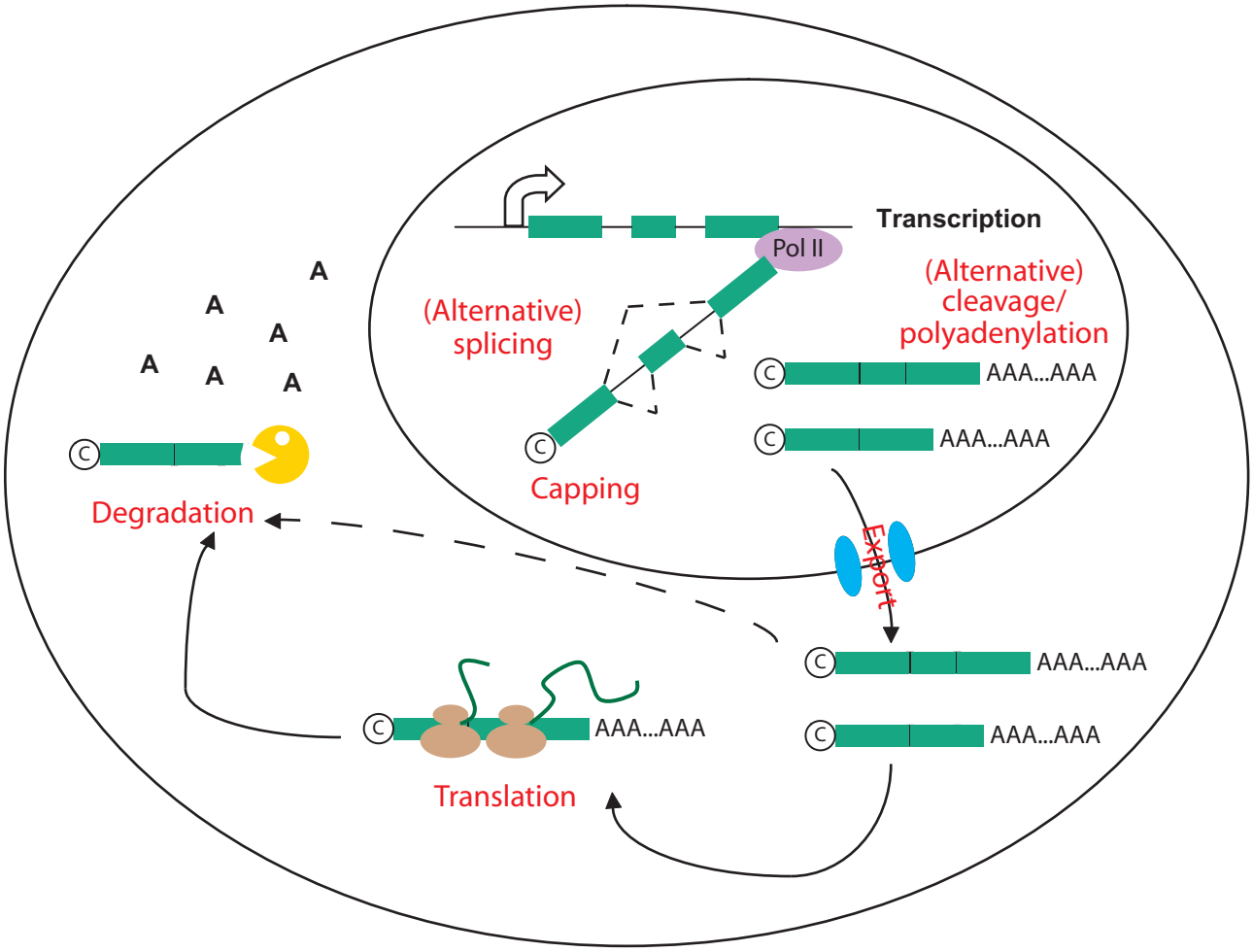


FIGURE OF BOX 3