

Post-transcriptional controls - adding a new layer of regulation to clock gene expression.

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

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

27

28 **Rhythmic gene expression in oscillators**

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

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

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

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

50 The mammalian circadian clock relies on eight proteins that are cyclically expressed in
51 the SCN (Figure 1A): Clock [7], Bmal1 (Mop3) [8], Per1, Per2 and Per3 [9], Cry1 and Cry2
52 [10], and Rev-Erb α [11]. The Clock-Bmal1 complex controls the expression of several genes
53 at the transcription level, among which *Period* (*Per1* to *Per3*), *Cryptochrome* (*Cry1* and
54 *Cry2*), and *Rev-Erb α* , through its association with E-box elements. The Per-Cry protein
55 complexes interact with and inhibit Clock-Bmal1, and Rev-Erb α inhibits the transcription of
56 *Bmal1*. These two transcriptional feedback loops are responsible for the oscillations of Clock-
57 Bmal1 activity that themselves account for the circadian expression of the clock outputs
58 (Figure 1A) [4,5]. Several additional factors that modulate the mammalian circadian clock
59 were recently identified by RNAi or proteomic approaches [12,13]. The circadian and the
60 segmentation (Box 2) clocks both are set-up by transcriptional negative-feedback loops [2,14-
61 18].

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

72

73 **Circadian rhythms as a paradigm for dynamic posttranscriptional controls**

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

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

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

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

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

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

126 ultradian rhythmic expression of several genes in mouse liver [35]. This might indicate some
127 ultradian clock, but an alternative cause could be mRNA degradation. If genes are transcribed
128 following circadian rhythms and the corresponding mRNAs are degraded following a
129 circadian, out-of-phase, rhythm, the mRNA levels might oscillate with a period of 12 hours
130 [35].

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

137

138 **Clues for the importance of posttranscriptional controls in biological rhythms**

139 How widespread are posttranscriptional controls of gene expression in biological
140 rhythms? A rough estimate is provided by identifying factors that control gene expression at
141 the posttranscriptional level and that display a rhythmic circadian expression. This is the case
142 for several miRNAs in the plant *Arabidopsis thaliana* [37], fly heads [38] and mouse retinas
143 [39].

144 Several examples of oscillating RNA-BPs have also been reported, in addition to the
145 factors described in the previous section. In the green alga *Chlamydomonas reinhardtii*, the
146 capacity of the RNA-binding complex CHLAMY1 to bind to target mRNAs follows a
147 circadian rhythm [40]. CHLAMY1 comprises two subunits that both are RNA-BPs.
148 Experimentally manipulating the level of either of these two subunits strongly interferes with
149 the circadian rhythm, suggesting that these two proteins are at the heart of the circadian clock
150 in this species [41]. The *Chlamydomonas* clock is entrained by temperature cycles, and both
151 subunits of CHLAMY1 are involved in temperature integration [42]. In rats, the RNA-BP
152 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

180 cause its degradation. Together with the capacity of FFC to repress the transcription of *frq*
181 gene, this posttranscriptional negative-feedback loop achieves circadian oscillations in *N.*
182 *crassa* [48]. In *Arabidopsis thaliana*, *AtGRP7* and *AtGRP8* are two RNA-BPs with a
183 circadian expression. *AtGRP7* overexpression ablates circadian expression of *Atgrp7* and
184 *Atgrp8* mRNAs [49]. Both proteins are able to bind to their own pre-mRNAs and direct their
185 splicing pathways towards mRNA isoforms that contain a premature termination codon.
186 These isoforms are rapidly degraded by the non-sense-mediated mRNA decay (NMD)
187 pathway (see Box 3). Consequently, *AtGRP7* and *AtGRP8* negatively auto-regulate and cross-
188 regulate their synthesis [50,51]. This mechanism very probably ensures a cyclical stability of
189 the mRNAs encoding *AtGRP7* and *AtGRP8*, which contributes to their circadian oscillations.

190 In mammals, the RNA-BPs Rbm4 and Syncrip display oscillating expressions [28,33].
191 It is tempting to speculate that these oscillations result from negative auto-regulations similar
192 to plant *AtGRP7* and *AtGRP8* or *N. crassa* FRQ. Indeed, several mammalian RNA-BPs
193 negatively regulate their own synthesis. PTBP1 and PTBP2 regulate the splicing of their own
194 respective pre-mRNAs and promote the skipping of an exon that results in an NMD sensitive
195 transcript [52,53]. They also cross-regulate each other through this splicing event [54,55].
196 Similarly, the RNA-BP Celf2 negatively autoregulates its synthesis by inhibiting the splicing
197 of its own pre-mRNA [56]. Whether these negative auto-regulations of RNA-BPs generate
198 oscillations, and how these putative posttranscriptional negative-feedback loops are
199 interconnected with the master transcriptional loop, have not been tested in mammals.

200

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

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

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

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

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

228 Reporter genes also showed that mRNA degradation is required to achieve the
229 dynamic expression pattern of the clock genes. In Zebrafish, a GFP reporter controlled by the
230 *Her1* promoter (an oscillating component of the core clock) accumulates in the presomitic
231 mesoderm owing to its high stability, suggesting *a contrario* the rapid decay of the
232 endogenous mRNA [59]. In *Xenopus* transgenic embryos, a characteristic striped expression
233 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

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

267

268 **Concluding remarks and future directions**

269 The comparison of the segmentation and circadian clocks paves the way for future
270 researches (Box 4). Both mRNA degradation and translation, mediated by RNA-BPs and
271 miRNAs, have recognized functions in the circadian clock. In several instances, translational
272 efficiency and mRNA degradation oscillate in the circadian clock, and these oscillations fully
273 contribute to the clock. By contrast, the only known mode of posttranscriptional control in the
274 segmentation clock is constant mRNA degradation. In fact, we might simply lack data
275 concerning the different modes of posttranscriptional controls in the segmentation clock.
276 Using the circadian clock as a paradigm for posttranscriptional controls in clocks, we
277 recommend that the various modes of oscillating posttranscriptional controls should be
278 carefully investigated in the segmentation clock. Furthermore, most but not all known modes
279 of posttranscriptional controls were described in the circadian clock. Specifically, we know
280 nothing about the subcellular localization and the putative localized translation of the mRNAs
281 encoding factors of the clock. It might be of interest to investigate these points in the
282 regulation of mammalian circadian clock considering their recognized importance in neurons
283 [73].

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

288 in somitic segmentation, most of which were not identified, will be potential candidates for
289 causing these syndromes. Also several human troubles arise from defects in the circadian
290 clock, such as sleep disorders. Interestingly, fragile X patients suffer from sleep disorders
291 [75]. This syndrome is a consequence of impaired expression of the RNA-BP FMR1, and
292 *Fmr1* KO mice display an altered circadian rhythm [76]. Fragile X syndrome provides
293 therefore a link between posttranscriptional controls, human pathology and the circadian
294 clock, and it can be anticipated that this will not remain an isolated example.

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

310

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316

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528 **Glossary box**

529 **3' Untranslated Region (3'UTR):** Region of the mRNA 3' to the translation stop codon.

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

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

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

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

544 **Infradian rhythm:** A cycle of length above 24h.

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

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

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

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

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

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

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

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

562 **Box 1. Some examples of biological rhythms**

563

564 Depending on the period, biorhythms are classified as ultradian (period $T < 24\text{h}$), infradian
565 ($T > 24\text{h}$) and circadian ($T \sim 24\text{h}$). Ultradian rhythms include heart beating ($T =$ fractions of
566 seconds to seconds), sleep episodes ($T =$ tens of minutes), respiratory oscillations in yeasts
567 ($T = 1\text{--}5\text{h}$ [83]), somitic segmentation in vertebrates ($T = 30$ minutes in Zebrafish, 2h in mice
568 [2]), or pulses of LH secretion by the pituitary gland ($T \sim 3\text{h}$ in men [84]). Infradian rhythms
569 include successions of torpor and arousal during the hibernation of small mammals
570 ($T =$ several days [85]), female menstrual cycles ($T =$ several days to several months), annual
571 rhythms (flowering of most plants), and even pluri-annual rhythms such as the emergence of
572 Cicada [86].

573 **Box 2. The vertebrate segmentation clock.**

574 Please refer to the accompanying figure.

575 Title of the figure "The zebrafish core segmentation clock"

576

577 In zebrafish, the core segmentation clock consists of Her1 and Her7 proteins (see Figure).

578 Homodimers or heterodimers of these proteins bind to their own promoters and repress their

579 transcription. Taking into account transcriptional and translational delays, this results in

580 oscillating levels of these proteins. Furthermore, Her1/7 duplexes repress the transcription of

581 Delta-C, a transmembrane Notch ligand. When bound by its ligand, the Notch transmembrane

582 receptor undergoes a limited proteolysis that releases the Notch intracellular domain (NICD)

583 in the cytoplasm. NICD is then translocated to the nucleus. Together with Su(H) protein, the

584 NICD stimulates the transcription of target genes including *Her1* and *Her7*. The stimulation

585 of *Her1/7* transcription by Delta-C expressed in adjacent cells, and the ensuing repression of

586 *Delta-C* gene by Her1/7 achieves coordinated oscillations in neighbouring cells [18,63].

587 Her13.2 reinforces the transcriptional inhibition mediated by Her1/7, and it is controlled by

588 the FGF pathway. This links the Notch and FGF signalling pathways [87]. Several other

589 genes are downstream of Her1/7 and are involved in somitic segmentation. In amniotes

590 (chick, mouse), the segmentation clock is more complex. It requires oscillations of the Notch

591 modulator Lunatic fringe, and of tens of mRNAs that encode proteins belonging to the FGF

592 and Wnt signalling pathways in addition to Notch [57].

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

594

595 Please refer to the accompanying figure.

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

597

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

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

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

629 **Box 4. Future questions**

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

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

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

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

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

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

648 **Figure legends**

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

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

665

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

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

Gene	Function in the clock	Evidence for posttranscriptional controls	References
<i>Llnfg</i> 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 <i>Her1</i>	Encodes component of the core clock	The expression pattern of a reporter mRNA controlled by <i>Her1</i> promoter is different from that of endogenous <i>Her1</i> due to increased mRNA stability. Expression pattern in the <i>Tortuga</i> mutant consistent with <i>Tortuga</i> gene product being responsible for <i>Her1</i> mRNA instability	[59,64]
<i>Xenopus Hairy 2a, Hairy 1, Esr5, Nrarp, Bowline, Chick Hairy 1, Mouse Hes1, human HES4</i>	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 <i>Xenopus</i> , the 3'UTR of <i>Hairy 2a</i> confers instability on a reporter mRNA. The expression pattern of <i>Hairy 2a</i> or <i>Bowline</i> 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]
<i>Xenopus Su(H)</i> (homologue of mammalian <i>Rbpj</i>)	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 <i>Su(H)</i> mRNA causes segmentation defects.	[65,67]

674

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

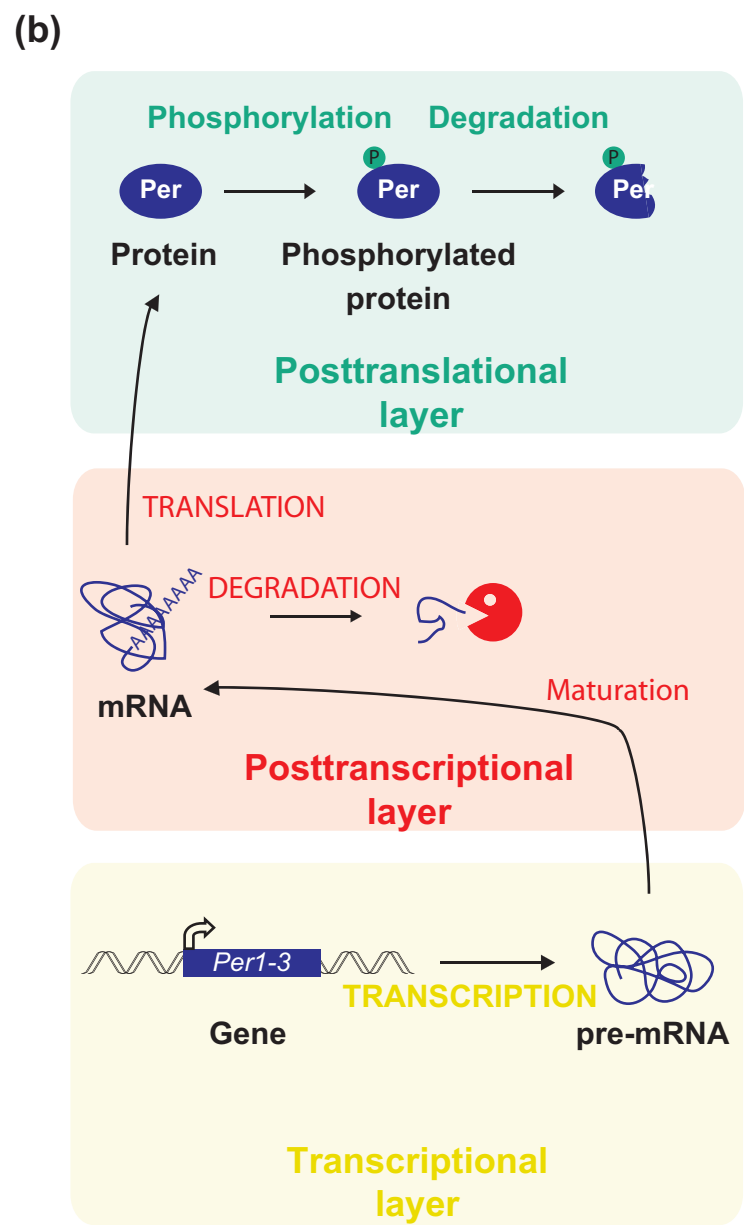
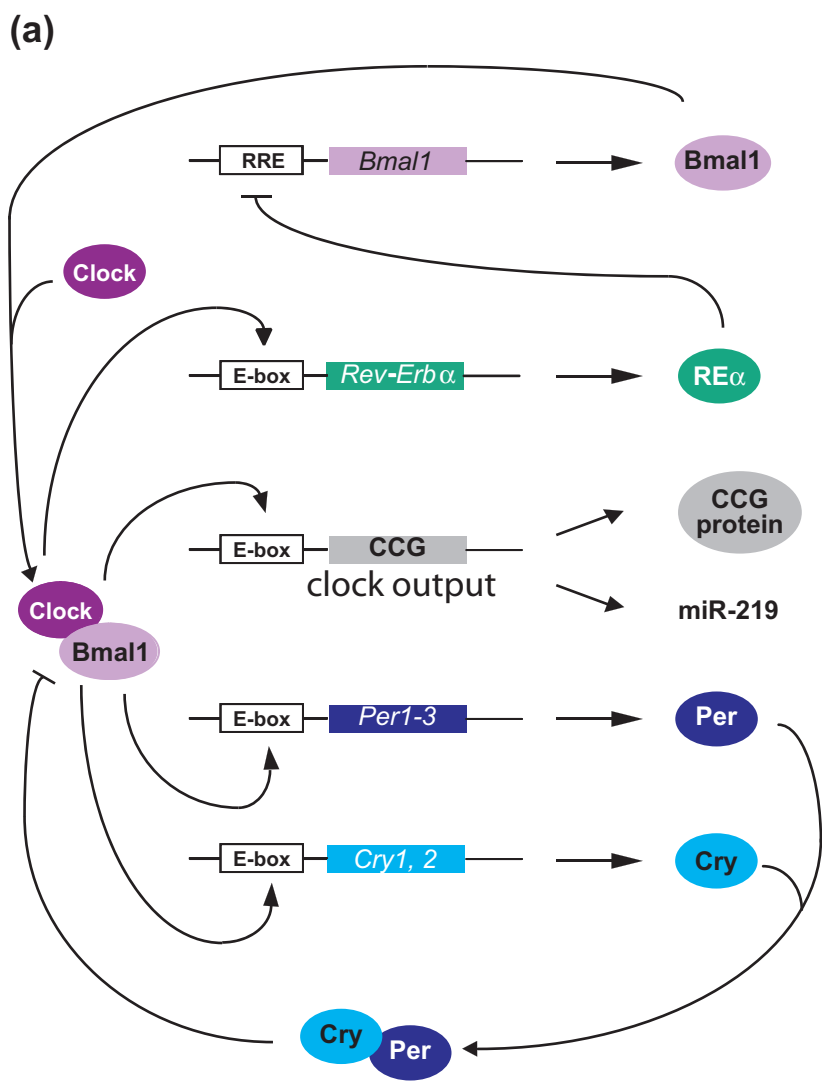


FIGURE 1

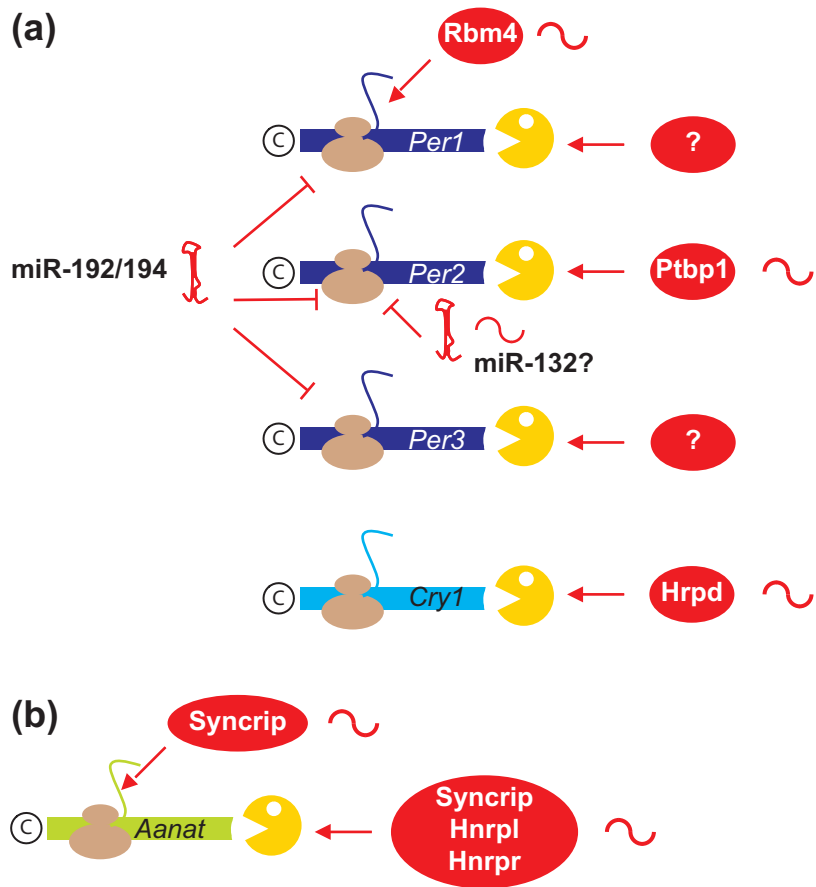


FIGURE 2

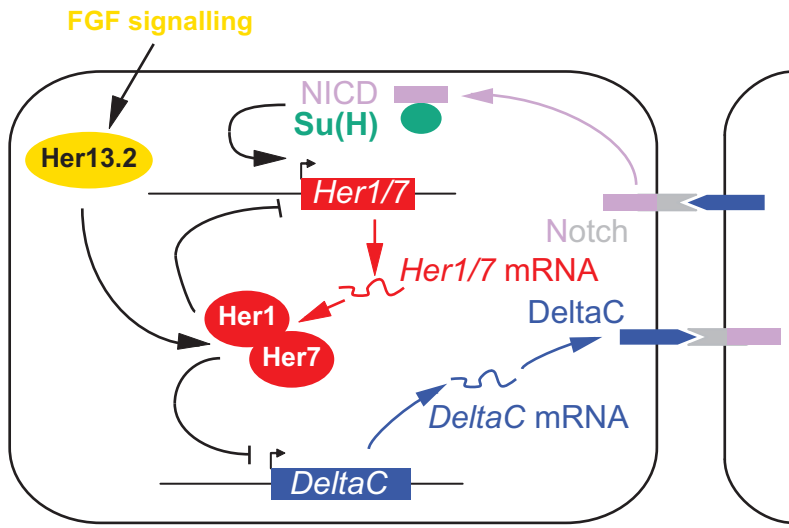


FIGURE OF BOX 2

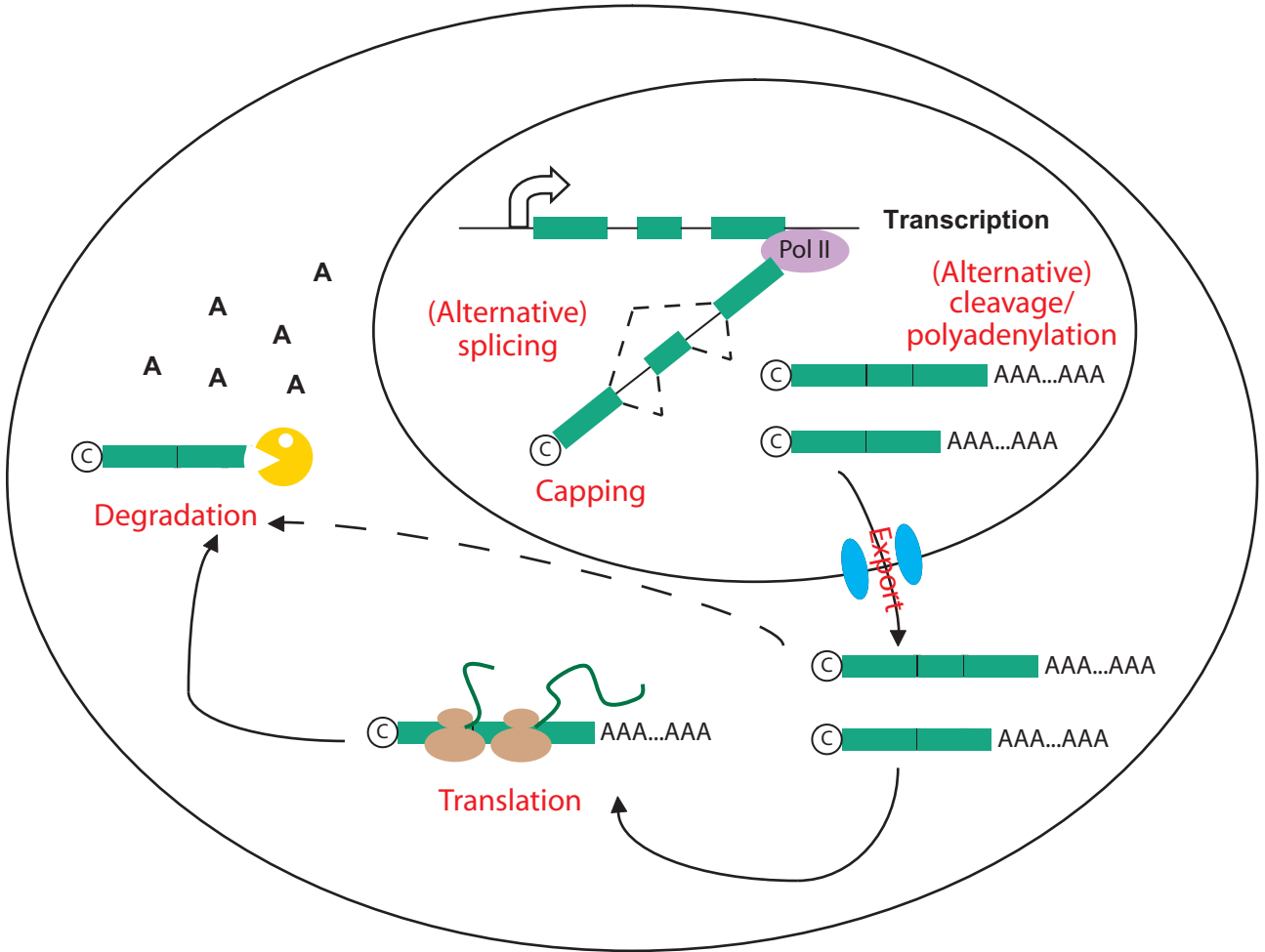


FIGURE OF BOX 3