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# Running after the clock

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**ABSTRACT The way we currently understand vertebrate development is undoubtedly associated with the research undertaken at the "Institut d'Embryologie Cellulaire et Moléculaire" at Nogent-sur-Marne during the last decades. Working in this Institute has been a privilege for many junior and senior researchers. Eight years ago, in this stimulating environment, an exciting observation followed by a series of revealing experiments gave rise to a novel field of research. This study provided evidence for the existence of a molecular clock underlying chick somite formation. In this review, we focus on the cascade of studies that have followed this discovery. Thus far, it has been demonstrated that the molecular clock is operating in several vertebrate models namely chick, mouse, zebrafish, frog and medaka, probably functioning to provide cells with multidimensional positional information. Loss and gain of function experiments and detailed gene promoter analyses have proved very useful in understanding how the clock machinery works. Recent data has also led to the fascinating hypothesis that the clock might not be an exclusive property of somitic cells, but rather a mechanism used by a wide range of embryonic tissues. Meanwhile, the clock "keeps ticking" and many questions are still waiting for an answer.**

KEY WORDS: *molecular clock, cycling gene, segmentation, somitogenesis, vertebrate embryo*

Segmentation is an evolutionary successful feature that starts early during embryonic development with the formation of transient metameric structures called somites. Somites will give rise to the segmented structures in the vertebrate embryo such as vertebrae, intervertebral disks, ribs and skeletal muscles. These structures provide an efficient protection to the internal vital organs, while conferring a high degree of mobility to the adult body. Somites form as epithelial spheres in an anterior (A) to posterior (P) sequential manner, bilateral to the axial midline of the embryo. Each somite buds off periodically from the most anterior tip of the unsegmented mesenchymal paraxial mesoderm or presomitic mesoderm (PSM) (reviewed by Gossler and Hräbe de Angelis, 1998). For a given species, temporal periodicity of somite formation is so remarkably precise that it has retained the attention of embryologists for many decades. Several theoretical models have tried to explain the precision of somitogenesis. Many aspects of the cellular and molecular mechanisms underlying this process have been unveiled in the last years although many fundamental questions remain to be addressed.

## Somite formation in the light of classical models

Three main classical models have been proposed to explain the periodicity of somite formation: the Meinhardt's model, the cell cycle model and the clock-and-wavefront model.

Meinhardt proposed that prior to the formation of each somite, presomitic cells undergo several oscillations between two alternate states corresponding to the prospective A and P somitic compartments (Meinhardt, 1986). As postulated, the confrontation between cells of incompatible A and P states would result in a physical boundary between consecutive somites, since there is no intermingling between cells from different states. Juxtaposition of these two segregated states would also lead to the formation of a physical barrier in the middle of a somite. To overcome this problem Meinhardt postulated a third oscillating state, called segment border (S), which corresponds to the somitic boundary. However, while distinct cell adhesive characteristics allowed the identification of A and P somitic compartments (Keynes and Stern, 1984), no S cells have been identified so far. In addition, a study using confocal time-lapse microscopy challenges the Meinhardt's model by showing that some degree of cell intermingling between A and P somitic compartments does occur during somite boundary formation (Kulesa and Fraser, 2002).

In 1988, Primmett and collaborators demonstrated that a single heat-shock applied to the chick embryo gives rise to several segmentation abnormalities that are repeated along the AP axis

*Abbreviations used in this paper:* EJM, ejemplo poner aquí ; QTR, quitar si no hay escribir desde aquí hacia arriba.

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with a regular interval of six to seven somites (Primmett *et al.*, 1988). The time required to form six to seven somites in the chick is approximately ten hours, which corresponds to the time necessary for the completion of the cell cycle in the PSM (Primmett *et al.*, 1989). Consequently, it was postulated that the cell cycle would function as an internal cellular clock related to the process of segmentation: cells located in the rostral PSM share some degree of cell cycle synchrony, increase their adhesive properties and thus assemble and give rise to a somite. However, no correlation between the duration of the cell cycle in PSM and the rate of somitogenesis, which takes 90 minutes in the chick embryo, has been found so far.

The clock-and-wavefront model postulated the existence of two independent phenomena accounting for the periodic somite formation (Cooke and Zeeman, 1976). On one hand, there is an intrinsic clock compelling presomitic cells to oscillate synchronously between a permissive and a non-permissive state. On the other hand and concomitantly, a wavefront travels along the embryonic axis establishing an AP gradient of differentiation. This model predicts that in order to form a somitic boundary, a group of PSM cells oscillating synchronously has to be reached by the wavefront of differentiation. Experimental data gathered so far seem to support both assumptions of the clock-and-wavefront model.

## The segmentation clock: the beginning of times

In 1997, Palmeirim and collaborators provided the first molecular evidence for the existence of an intrinsic oscillator operating in the presomitic cells of chick embryos. They observed that, within groups of embryos with the exact same number of somites, the basic Helix-Loop-Helix (bHLH) transcription repressor *hairy1* displayed remarkably different patterns of expression in the PSM. The authors have also demonstrated that this dynamic expression is reiterated every 90 minutes, corresponding exactly to the time required to form a pair of somites. Moreover, these mRNA oscillations were shown to be an autonomous property of PSM cells and neither a consequence of cell migration nor dependent on a diffused signal within the PSM. This pioneer study has demonstrated that presomitic cells undergo several periodic oscillations of the *hairy1* gene expression before they incorporate a somite, conceptually describing a caudal wave that progresses anteriorly and stabilises in a narrow stripe in the rostral PSM (Palmeirim *et al.*, 1997).

After the discovery of the *hairy1* gene in the chick embryo, many other genes have also been reported to have a cyclic expression at the level of the PSM, suggesting that the segmentation clock involves a complex genetic network. It is now clear that the molecular mechanism underlying somitogenesis is highly conserved among vertebrates, since periodic gene transcription has also been described in mouse, zebrafish, frog and medaka. The majority of the cycling genes code for Hairy/Enhancer-of-Split (Hes) family targets of the Notch signalling pathway such as *hairy1* and *hairy2* in the chick (Palmeirim *et al.*, 1997 and Jouve *et al.*, 2000), *hey2* both in chick and mouse (Leimeister *et al.*, 2000), *hes1* and *hes7* in mouse (Jouve *et al.*, 2000; Bessho *et al.*, 2001b), *her1* and *her7* in zebrafish (Holley *et al.*, 2000; Oates and Ho, 2002;) *esr9* in the frog (Li *et al.*, 2003) and *her7* in medaka (Elmasri *et al.*, 2004). Other cycling genes encode a modulator of the Notch signalling pathway, *lunatic fringe* (*lfng*), in the chick and mouse, (McGrew *et al.*, 1998; Aulehla and Johnson, 1999; Forsberg *et al.*, 1998) and a Notch ligand, *deltaC*, in zebrafish (Jiang *et al.*, 2000). Furthermore, work by Aulehla and colleagues (2003) has shown that a repressor of the Wnt signalling pathway, *axin2*, is also cycling in the mouse PSM (Aulehla *et al.*, 2003) (Table 1). More recently, nkd1, a wnt antagonist, has also been shown to exhibit an oscillatory expression pattern in the mouse PSM, suggesting a reciprocal interaction of Notch and Wnt signals in the regulation of the segmentation clock (Ishikawa *et al.*, 2004).

## Looking at the mutants

The fact that the majority of the cycling genes code for components of the Notch signalling pathway suggests that it plays a role in the segmentation clock. Indeed, the analysis of *notch1* (Conlon *et al.*, 1995), *delta-like1* (*dll1*), *delta-like3* (*dll3*) (Hräbe de Angelis *et al.*, 1997; Kusumi *et al.*, 1998), *lunatic fringe* (*lfng*) (Zhang and Gridley, 1998; Evrard *et al.*, 1998), *presenilin1* (Wong *et al.*, 1997), *rbp-jk* (Oka *et al.*, 1995), *pofut1* (Shi and Stanley, 2003) and *hes7* (Bessho *et al.*, 2001b) mutants reveals a somitic phenotype. In general these defects consist of disrupted AP segment polarity, misaligned and misshapen caudal somites, while more rostral somites seem to be less affected. Although most of these mutations are lethal, the analysis of the embryos at early stages reveals defects in the organisation of the sclerotome and dermomyotome, eventually leading to severe perturbations in the axial skeleton. Thus far, both *hes1* and *hes5* are the only Notch signalling mutants without a somitic phenotype (Jouve *et al.*, 2000; Ohtsuka *et al.*, 1999). These results reflect a possible compensation by other cyclic *hes* genes such as *hes7* (Bessho *et al.*, 2001a). In zebrafish, several somite mutants have been isolated from a large-scale screening and the detailed analysis of their phenotypes shows a striking resemblance to the Notch signalling mouse mutants (Jiang *et al.*, 1996; Van Eeden *et al.*, 1996). In fact, it is now known that the phenotypes of the zebrafish mutants *deadly seven* (*des*), *after*

TABLE 1

**CYCLIC GENES REPORTED IN VERTEBRATE SPECIES**

| Chick | Mouse | Zebrafish | Frog | Medaka |
|---|---|---|---|---|
| *hairy1* (Palmeirim *et al.*, 1997) | *hes1* (Jouve *et al.*, 2000) | *her1* (Holley *et al.*, 2000) | *esr9* (Li *et al.*, 2003) | *her7* (Elmasri *et al.*, 2004) |
| *hairy2* (Jouve *et al.*, 2000) | *hes7* (Bessho *et al.*, 2001b) | *her7* (Oates *et al.*, 2002) | | |
| *hey2* (Leimeister *et al.*, 2000) | *hey2* (Leimeister *et al.*, 2000) | *deltaC* (Jiang *et al.*, 2000) | | |
| *lunatic fringe* | *lunatic fringe* | | | |
| (McGrew *et al.*, 1998; | (Forsberg *et al.*, 1998) | | | |
| Aulehla and Johnson, 1999) | *axin2* (Aulehla *et al.*, 2003) | | | |
| | *ndk1* (Ishikawa *et al.,* 2004) | | | |

*eight* (*aei* ) and *mind bomb* (*mib* ) are due to null mutations in *notch1*, *deltaD* and in an ubiquitin ligase that binds Delta, respectively (Holley *et al.*, 2002; Holley *et al.*, 2000; Itoh *et al.*, 2003). Injection of morpholinos targeted for these genes recapitulates the phenotype of each of these zebrafish mutants (Holley *et al.*, 2002; Itoh *et al.*, 2003). Although there are no zebrafish mutants for the *deltaC*, *her1*, *her7* and *suppressor of hairless* (*su* (*h* )) genes, morpholino knockdown experiments have revealed that, in the absence of the proteins coded by these genes, the embryos exhibit a somitic phenotype (Holley *et al.*, 2002; Henry *et al.*, 2002; Oates and Ho, 2002; Gajweski *et al.*, 2003; Sieger *et al.*, 2003). More recently, it has also been described that the zebrafish Her6 is an output of the Notch signalling pathway that, together with Her4, is required for maintaining the synchronization of cyclic gene expression within PSM (Pasini *et al.*, 2004). Similarly, cyclic gene expression in the PSM is lost by reducing the receptor protein tyrosine phosphstase ψ (RPTP ψ) using morpholino antisense oligonucleotides, suggesting a requirement for RPTP ψ in the control of the clock upstream of, or in parallel with, Delta/Notch signalling (Aerne and Ish-Horowicz, 2004) (Table 2).

Despite the somitic defects observed in the mouse and zebrafish mutants, the sclerotome and dermomyotome present a more or less organised segmental pattern, indicating that a basic metameric pattern is accomplished in the somitic derivatives. It seems that the Notch signalling pathway is important to coordinate the periodicity of somite formation and to specify somitic AP polarity, although its downregulation is not sufficient to abolish overall segmentation. This could be due to the fact that Notch activation is not the only determinant responsible for the formation of segments or because there is a certain degree of redundancy between Notch signalling components.

Phenotypic analysis of Notch signalling mutants strongly suggested that the defects observed could be due to a disruption in molecular segmentation and, therefore, the expression pattern of the cycling genes was studied in these mutants.

The expression of *lfng* is downregulated in *dll1*, *dll3*, *pofut1* and *rbp-jk* mutant mice but it is only slightly reduced in the *notch1* mutant (Barrantes *et al.* 1999; Shi and Stanley, 2003). On the contrary, the cyclic behaviour of *lfng* is not affected in *hes1* knockout mice (Jouve *et al.*, 2000). In *hes7* null mice *lfng* transcription is constitutively upregulated in all presomitic cells (Bessho *et al.*, 2001b). The *hes1* gene expression is severely downregulated in *dll1*, *dll3* and *hes7* homozygous null embryos (Jouve *et al.*, 2000; Dunwoodie *et al.*, 2002; Bessho *et al.*, 2001b). Moreover, the transcription of *hey2* is also downregulated in the PSM of *hes7* knockout mice (Bessho *et al.*, 2001b). Additionally, the transcription of *hes7* is constitutively upregulated in *hes7* mutants, as demonstrated by the expression of intronic probes in the PSM (Bessho *et al.*, 2003).

In zebrafish, the cyclic expression of *her1*, *her7* and *deltaC* is impaired in the *aei* (DeltaD), *des* (Notch1) and *mib* (Ubiquitin ligase) mutants and in the *su* (*h* ) morpholino-knockdown experiments (Holley *et al.*, 2000; Holley *et al.*, 2002; Oates and Ho, 2002; Jiang *et al.*, 2000; Sieger *et al.*, 2003). Additionally, *her1* is impaired in *deltaC* morpholino injected embryos (Holley *et al.*, 2002). The inhibition of Her1 function in the PSM leads a loss of the cyclic expression of both *her1* and *deltaC*. In this knockdown experiment *her7* expression is decreased but its cyclic behaviour is maintained (Holley *et al.*, 2002; Oates and Ho, 2002). A decrease in Her7 function disrupts the dynamic expression of *deltaC*, *her1* and *her7* (Oates and Ho, 2002).

Overall, these studies show that the oscillations of the cycling genes are in fact disturbed in Notch signalling mutants, reaffirming the function of the Notch pathway in driving the segmentation clock and showing that this role is conserved among vertebrates. Jiang and colleagues (2000) observed that while in normal development PSM cells oscillate synchronously, in Notch signalling mutants they drift out of synchrony eventually leading to defective somitogenesis. The authors show that in Notch signalling zebrafish mutants, the expression pattern of *deltaC* is normal at first, but becomes desynchronised, which could account for the sparing of the first somites in these mutants. Thus, it was proposed that the essential function of the Notch signalling pathway is to maintain the oscillations synchronised in adjacent PSM cells (Jiang *et al.*, 2000).

## Dissecting clock promoters

As discussed above there are several PSM genes that show a cyclic expression pattern, however the mechanism that generates this pattern is not completely understood. In the mouse, it was shown that the cyclic expression of *lfng* in the PSM is controlled at the level of transcription by periodic activation of its promoter (Morales *et al.*, 2002). Analysis of the *lfng* promoter by successive deletions of the 5'UTR sequence led to the identification of a *cis* -regulatory region that is able to recapitulate the cyclic expression of this gene. A human equivalent region also drives the cyclic expression of a reporter gene in the mouse PSM (Cole *et al.*, 2002; Morales *et al.*, 2002). Comparison of both human and mouse *cis* -regulatory regions disclosed an evolutionary conserved 110 kilobase fragment that is a strong candidate to regulate the periodic gene

TABLE 2

**LOSS OF FUNCTION OF NOTCH SIGNALLING PATHWAY COMPONENTS INVOLVED IN SOMITOGENESIS**

| Mouse mutants | Zebrafish mutants |
|---|---|
| ***With a somitic phenotype*** | *notch1 - deadly seven (des)* (Holley *et al.*, 2002) |
| *hes7* (Bessho *et al.*, 2001b) | *deltaD - after eight (aei)* (Holley *et al.*, 2000) |
| *notch1* (Conlon *et al.*, 1995) | *ubiquitin ligase - mind bomb (mib)* (Itoh *et al.*, 2003) |
| *rbp-jk* (Oka *et al.*, 1995) | |
| *presenilin1* (Wong *et al.*, 1997) | **Zebrafish morphants** |
| *delta-like1* (Hräbe de Angelis *et al.*, 1997; | *her1*$^{mo}$ (Holley *et al.*, 2002; Henry *et al.*, 2002; |
| Cordes *et al.*, 2004) | Oates and Ho, 2002; Gajewski *et al.*, 2003) |
| *delta-like3* (Kusumi *et al.*, 1998) | *her7*$^{mo}$ (Oates and Ho, 2002; Henry *et al.*, 2002; |
| *lunatic fringe* (Zhang and Gridley, 1998; | Gajewski *et al.*, 2003) |
| Evrard *et al.*, 1998) | *her1*$^{mo}$+*her7*$^{mo}$ (Oates and Ho, 2002; Henry *et al.*, 2002) |
| *pofut1* (Shi and Stanley, 2003) | *notch1*$^{mo}$ (Holley *et al.*, 2002) |
| | *suppressor of hairless*$^{mo}$ (Sieger–*et al.*, 2003) |
| ***Without a somitic phenotype*** | *deltaC*$^{mo}$ (Holley *et al.*, 2002) |
| *hes1* (Jouve *et al.*, 2000) | *deltaD*$^{mo}$ (Holley"*et al.*, 2002) |
| *hes5* (Ohtsuka *et al.*, 1999) | *her4*$^{mo}$ (Pasini et al., 2004) |
| | *her6*$^{mo}$ (Pasini et al., 2004) |
| | *her6*$^{mo}$ + *her4*$^{mo}$ (Pasini et al., 2004) |
| | *RPTP* $^{mo}$ (Aerne and Ish-Horowicz, 2004) |

$^{mo}$ - morpholino

oscillations. Further dissection of this fragment has shown that it contains two E-boxes, which are binding sites for bHLH proteins, as well as a binding site for the CBF1 (Rbp-Jk; Su(H)) transcription factor (Cole *et al.*, 2002; Morales *et al.*, 2002). So it seems that cyclic bHLH Notch targets may regulate the segmentation clock acting as transcriptional repressors that bind to the E-boxes in the *cis* -regulatory region. Indeed, Hes7 binds E-boxes and thus it was proposed to mediate the cyclic repression of *lfng* (Bessho *et al.*, 2003). Mutations in the CBF1 binding site lead to a decrease in *lfng* expression in the mouse PSM (Cole *et al.*, 2002; Morales *et al.*, 2002), which indicates that this region functions as an activator element within the promoter. In addition, these experiments also demonstrate that the expression of *lfng* is distinctly regulated in the anterior and the posterior part of the PSM, which is in agreement with previous work performed in the frog and the fish (Jen *et al.*, 1999; Holley *et al.*, 2002). Analysis of the *her1* promoter in the zebrafish embryo has also demonstrated that a 5'UTR fragment is able to drive its dynamic expression in the PSM. This study also pointed to the existence of regulatory elements that distinctly control *her1* expression in either the anterior or the posterior PSM (Gajewski *et al.*, 2003). A complex interaction between repressor and activator clock elements in the promoter seems to be a general requirement for the oscillatory behaviour of segmentation genes.

## Unveiling the clock mechanism

A negative feedback loop is a mechanism by which the expression of a gene is repressed by its own protein product. It has been suggested by several authors that the mechanism that drives the oscillations of the segmentation genes relies indeed on feedback inhibition.

The first direct evidence for the molecular mechanism that generates the oscillatory behaviour of the cyclic genes was presented in a study performed in cell culture (Hirata *et al.*, 2002). The authors demonstrated that not only *hes1* mRNA, but also Hes1 protein, undergo oscillations of expression with the same periodicity as somite formation. These oscillations are produced by a negative feedback loop in which Hes1 protein periodically represses its own transcription. This study suggested that a similar mechanism could be responsible for the transcriptional oscillations generated by the segmentation clock (Hirata *et al.*, 2002).

Recently, it was shown that in the mouse embryo both *hes7* mRNA and Hes7 protein oscillate in the PSM (Bessho *et al.*, 2003). Hes7 protein localisation domains do not overlap with the regions where *hes7* mRNA is expressed. Moreover, transcription of *hes7* is constitutively activated in the absence of Hes7 protein and downregulated following stabilization of Hes7 protein. Therefore, Hes7 oscillations in the PSM rely on a negative autoregulatory loop (Bessho *et al.*, 2003). Accordingly, Hes7 protein instability was shown to be crucial for cyclic gene expression (Hirata *et al.*, 2004). Also in the mouse embryo, it was shown that *hes1* mRNA oscillations are blocked in the absence of a functional Hes1 protein, suggesting that this protein might regulate its own promoter (Hirata *et al.*, 2002). In zebrafish, the cyclic genes *her1* and *her7* seem to negatively regulate their own expression, although there is no data regarding their protein expression in the PSM (Holley *et al.*, 2002; Oates and Ho, 2002).

Another negative feedback loop involving the periodic production of Lfng protein was described in the chick PSM (Dale *et al.*, 2003). In addition to *lfng* mRNA, Lfng protein cycles with the same periodicity as somite formation. Furthermore, overexpression of *lfng* in the chick PSM impairs the cyclic expression of the Notch downstream targets, *hairy1*, *hairy2* and endogenous *lfng*. Since Lfng is a modulator of Notch activity, it seems that the oscillations of segmentation genes are due to periodic inhibition of Notch activation (Dale *et al.*, 2003). Studies performed in zebrafish and mouse revealed that the function of Lfng might be different from the one in the chick. In zebrafish, *lfng* mRNA does not oscillate in the PSM (Prince *et al.*, 2001; Leve *et al.*, 2001) and it seems that the periodic activation of Notch is undertaken by the cyclic gene *deltaC* (Jiang *et al.*, 2000). In contrast, *lfng* oscillates in the mouse PSM (Forsberg *et al.*, 1998), although its constitutive expression does not abolish cyclic expression of endogenous *lfng* (Serth *et al.*, 2003). This implies that the activity of Notch alone cannot be the only determinant of cyclic gene expression in the mouse embryo.

An interesting finding that might explain the Lfng results in the mouse is the role recently attributed to the Wnt signalling pathway as a regulator of the clock by acting upstream of the Notch pathway (Aulehla *et al.*, 2003). It was shown that *axin2*, a negative regulator of the Wnt pathway, is expressed in the PSM in a cyclic fashion. In the *vestigial tail* mutant mouse, which is a hypomorphic mutant of *wnt3a,* there are caudal segmentation defects and, interestingly, *axin2* is not expressed. Thus, a negative feedback loop was proposed in which Wnt3a induces *axin2* expression and then Axin2 negatively modulates Wnt signalling. Since *axin2* mRNA oscillates alternately with *lfng* mRNA, it was suggested that Wnt and Notch pathways interact antagonistically through the binding of *dishevelled* to the Notch intercellular domain (Aulehla *et al.*, 2003). Recently, it was shown that the Wnt-responsive transcription factor Lef1 binds to several sites in the *dll1* promoter and enhancer regions and regulates its activity in the mouse PSM. This finding establishes a molecular link between the Wnt and the Notch pathways during somitogenesis (Galceran *et al.*, 2004). So far, the involvement of Wnt signalling in the regulation of periodicity of somite formation has not been reported in other vertebrate embryos.

It is possible to consider the existence of three types of negative feedback loops by incorporating data from zebrafish, chick and mouse, although the possible interactions between these loops are still not understood: 1) a direct feedback loop that generates the cyclic expression of Hairy/ Enhancer-of-Split family of bHLH repressors (Her1 and Her7 in zebrafish and Hes1 and Hes7 in the mouse); 2) an indirect feedback loop that establishes periodic activation of Notch signalling (DeltaC in zebrafish and Lfng in the chick); 3) another indirect feedback loop that promotes periodic activation of Wnt signalling (Axin2 in the mouse) (Fig. 1).

It has been suggested that a negative feedback loop in which the expression of a gene is repressed by its own protein product would be insufficient to generate/maintain sustained oscillations (Hirata *et al.*, 2002). However, two mathematical models based on the experimental data from zebrafish and cell culture studies show that mRNA and protein oscillations can be produced if transcriptional and translational delays are taken into account (Lewis, 2003; Monk, 2003). Nevertheless, delayed feedback will only set up the pace of oscillations if the mRNA and protein half-

lives are effectively small in relation to the delay. Surprisingly, these mathematical models reveal that delay-driven oscillations are very resistant to parameter changes (Lewis, 2003; Monk, 2003). Simulating a reduction in the rate of protein synthesis revealed no effect on the oscillation period (Lewis, 2003), which could explain the occurrence of *hairy1* oscillations in the chick PSM after cycloheximide treatment (Palmeirim *et al.*, 1997). Moreover, a simulation where Notch signalling is impaired shows a progressive failure in the regularity of the oscillations which, in agreement with the desynchronization theory, could explain the mild defects in the first somites of zebrafish and mouse Notch mutants (Lewis, 2003; Jiang *et al.*, 2000).

## The wavefront: a partner of the clock

The clock-and-wavefront model proposes an explanation for the temporal and spatial regulation of somitogenesis. It predicts the existence of an intrinsic oscillator operating in parallel with a wavefront of differentiation, whose progression rate determines the correct positioning of somitic boundaries (Cooke and Zeeman, 1976). As previously discussed, the molecular evidence for the oscillator was provided by the cyclic expression of a number of genes, whereas the wavefront position seems to be regulated by Fgf and Wnt signalling (Dubrulle *et al.*, 2001; Sawada *et al.*, 2001; Aulehla *et al.*, 2003), by retinoic acid (RA) signalling and possibly by an unknown pathway involving the T-box gene, *tbx24* (Diez del Corral *et al.*, 2003; Nikaido *et al.*, 2002).

In the chick, *fgf8* defines a decreasing caudal to rostral gradient of expression in the two posterior thirds of the PSM (Dubrulle *et al.*, 2001). AP inversion experiments of PSM tissue demonstrated that AP somitic compartments are already determined in the anterior third of the PSM, in contrast to the caudal two thirds of this tissue where segment polarity is still undetermined (Dubrulle *et al.*, 2001). The transition between these two regions occurs at the level of the so-called determination front that progressively regresses as a consequence of embryo elongation and seems to correspond to the anterior limit of the *fgf8* gradient of expression. Either inhibiting or overexpressing Fgf8 at the level of the determination front alters the position of somitic boundaries, inducing the formation of larger or smaller somites, respectively. It seems that Fgf8 maintains posterior PSM cells in an immature state, thus negatively regulating the wavefront of differentiation in the chick embryo. Caudal to the determination front, the axial identity of PSM cells is also undetermined, since Fgf8 overexpression can induce an anterior shift of *hoxB9* and *hoxA10* expression domains (Dubrulle *et al.*, 2001). Additionally it was proposed that an interaction between the segmentation clock and the *hox* genes would establish the correct coordination between sequential segment formation and AP specification (Zakany *et al.*, 2001). Reinforcing this idea, transgenic mice expressing a dominant negative version of *Delta1* in the PSM showed subtle changes of *Hox* gene expression and alterations of vertebral identity resembling homeotic transformations (Cordes *et al.*, 2004).

The involvement of Fgf signalling in the control of the wavefront progression was also studied in zebrafish embryos (Sawada *et al.*, 2001). Fgf/mitogen-activated protein kinase (MAPK) signalling is functioning in the posterior PSM and it maintains these cells in an immature state. Experimental manipulations of MAPK levels in the PSM also lead to a variation in somitic size, strengthening the idea that Fgf signalling determines the position of segment border formation (Sawada *et al.*, 2001).

In the mouse, *wnt3a* seems to play a similar role to the one attributed to Fgf signalling in both chick and zebrafish PSM (Aulehla *et al.*, 2003). *wnt3a* is strongly expressed in the tail bud and it was proposed to establish a decreasing caudal to rostral gradient of expression that regresses as the embryo elongates. Furthermore, *fgf8* is downregulated in the tail bud and PSM of *wnt3a* hypomorphic mutants, suggesting that *wnt3a* acts upstream of *fgf8* in the regulation of the wavefront position. Since there is evidence that Fgf signalling may enhance Wnt/β-catenin signalling, Fgf8 might act as a relay enhancer of Wnt signalling in the PSM of mouse embryos (Aulehla *et al.*, 2003).

Previous studies have shown that presomitic tissue represses neuronal differentiation and that Fgf signalling can mimic this action (Diez del Corral *et al.,* 2002). On the other hand, the somitic mesoderm promotes maturation events, which are correlated with the activation of RA signalling in rostral PSM and somites, as indicated by the expression of the RA-synthesizing enzyme *raldh2* (Diez del Corral *et al.,* 2002 and 2003). Furthermore, explant culture experiments using a RA agonist (TTNPB) and Vitamin A deficient (VAD) quail embryos, which lack biologically

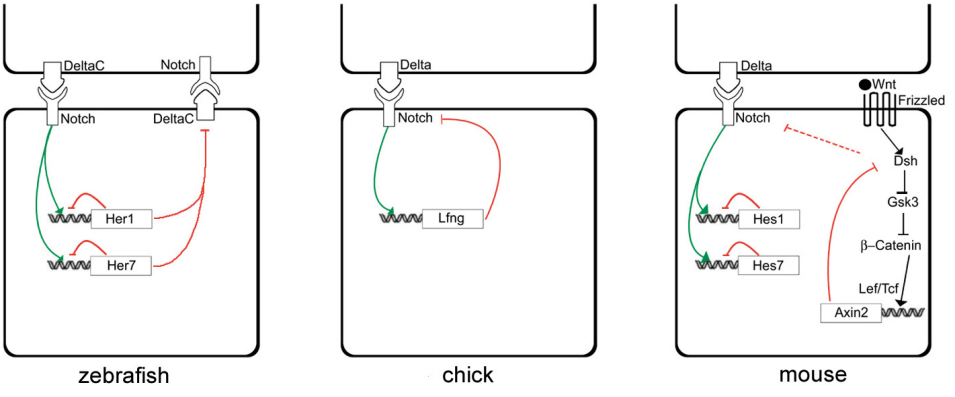


**Fig. 1. Feedback loops underlying periodic oscillations of cycling genes.** *In zebrafish, the periodic oscillations of* her1 *and* her7 *mRNA are generated by autoregulatory feedback loops and may be involved in* deltaC *periodic expression DeltaC in turn would periodically modulate Notch signalling activity. In the chick, Lfng protein indirectly represses its own expression by periodic modulation of the Notch signalling pathway. In the mouse, cyclic transcription of* hes7 *is regulated by the periodic expression of its own protein. The same molecular feedback loop generates Hes1 oscillations in different cell types. Additionally, the cyclic expression of* axin2*, a direct target of the Wnt pathway, regulates the Wnt signalling by a mechanism of feedback inhibition. It has been proposed that Wnt and Notch signalling pathways antagonistically interact since transcription of* axin2 *and* lfng *oscillates out of phase in the mouse PSM.*

active RA, have demonstrated that RA downregulates the expression of *fgf8* in the PSM (Diez del Corral *et al.,* 2003; Maden *et al.*, 1996). Conversely, Fgf8 soaked beads placed in the chick PSM represses the expression of *raldh2*, which indicates that Fgf signalling regulates the onset of RA synthesis in presomitic tissue (Diez del Corral *et al.,* 2003). These results show that Fgf and RA signalling pathways are mutually inhibitory and point to an important role of RA in inducing PSM cells maturation, in opposition to Fgf8. Future research will be important to reveal a possible interaction between the cycling genes (segmentation clock) and RA (wavefront) and their role in the control of somitogenesis.

The *fused somites* (*fss* ) zebrafish mutant lacks all somitic boundaries and this phenotype is the result of a mutation in the *tbx24* gene (Van Eeden *et al.*, 1996; Nikaido *et al.*, 2002). This suggests the involvement of *tbx24* in the formation of somitic boundaries. Although *tbx24* is expressed in anterior and intermediate PSM, its function seems to be restricted to the rostral PSM. In fact, in *fss* mutants segmentation genes specifically expressed in the anterior PSM are downregulated and the anterior stripe of the *her1* cyclic gene is lost, whereas its expression appears to be normal in the posterior PSM. These data suggest that *tbx24* plays a role in the maturation process of anterior PSM cells and that it might be independent of the molecular clock.

Fgf and Wnt signalling pathways seem to control the positioning of the wavefront of differentiation (Dubrulle *et al.*, 2001; Sawada *et al.*, 2001; Aulehla *et al.*, 2003). Since the formation of somitic boundaries is such a finely tuned process it is conceivable that a signal in the anterior PSM controls the precise site of the determination front. This signal could work in combination with the Fgf and Wnt posterior gradients that maintain cells in an immature state. As discussed above, both RA and *tbx24* are good candidates for this signal. Since *tbx24* has only been described in zebrafish, it would be interesting to look for functional homologues in other vertebrate models and to understand how the T-box pathway interacts with Fgf and Wnt signalling pathways.

## The clock in two dimensions

It is now well established that an intrinsic oscillator operates in PSM cells as it is revealed by the periodic expression of the cyclic genes. It seems that presomitic cells perceive the number of oscillations they undergo before incorporating a somite. This suggests that the segmentation clock constitutes a mechanism that provides AP positional information to these cells, determining their spatial organisation within the PSM (Palmeirim *et al.*, 1997). In the chick, a detailed analysis performed from stage 4 to 7HH (Hamburguer and Hamilton, 1951) showed that the cycling genes are expressed in the prospective somitic territory (Jouve *et al.*, 2002). This implies that presomitic cells are provided with their future AP positional information well before the first somite is formed.

In six somite stage chick embryos (stage 9- HH) a dynamic pattern of the cycling genes is also evident at the level of the presumptive presomitic territory, defining in this region an AP gradient of expression. A detailed quail-chick chimera fate map has revealed an anterior region located within this prospective territory that specifically gives rise to the medial part of the PSM and somites (Freitas *et al.*, 2001). Therefore, the AP gradient of expression in the prospective PSM territory describes a wave spreading throughout the future medial/lateral (ML) presomitic axis. Accordingly, a more careful analysis of expression pattern of the cycling genes at the level of the PSM unveils a ML asynchrony that is evidenced by oblique stripes of gene expression corresponding to a transition between two horizontal stripes (see supplemental data at: http://www.ijdb.ehu.es/ijdb200549023/49023esm317.mov). Thus, the segmentation clock is providing cellular positional information in at least two dimensions: not only along the AP but also along the ML presomitic axis (Freitas *et al.*, 2001).

## Final remarks

The development of a living organism is highly regulated in space and time. The only biological clock known to operate during embryonic development is the segmentation clock underlying the highly coordinated process of somite formation in vertebrates. However, the nature of the signal that triggers the molecular clock, early in development, remains to be determined. The segmentation clock is currently perceived as a cross talk between Notch and Wnt signalling cascades, consisting of complex regulatory feedback loops that ultimately generate periodic gene oscillations in presomitic cells. The cyclic transcription will only be translated into visible periodic gene expression patterns if both 1) the half-life of the mRNA is shorter than its transcription cycle and 2) a group of neighbouring cells is synchronous. It is possible that other genes undergoing cyclic transcription are not detected and so, their existence cannot be ruled out.

Recently, it was shown that the involvement of the Notch signalling pathway in the process of segmentation is not exclusive to vertebrates since it has been shown to be essential for the formation of segments in spiders (Stollewerk *et al.*, 2003). Such a finding suggests that a common ancestor of both vertebrates and arthropods might have used the same molecular mechanism for segmentation. Therefore, the fact that *Drosophila* does not use the Notch signalling pathway to make segments suggests that arthropods that generate segments simultaneously might have lost this ancestral segmentation strategy during evolution. It remains to be clarified whether the Notch signalling pathway is used by all sequentially segmented arthropods as well as whether a clock-like mechanism drives the cyclic transcription of Notch targets in these organisms.

It is now evident that a conserved clock mechanism dictates the timing of segment formation. At this point, an interesting question that arises is whether this clock operates in tissues other than presomitic mesoderm. As a matter of fact, an *in vitro* study has shown that several mouse cell lines express *hes1* mRNA and protein in a cyclic fashion. This, points to the possibility that different types of cells might measure time using a similar clock mechanism (Hirata *et al.*, 2002). Work from our group has provided evidence for cyclic *hairy2* expression in the chick limb bud, indicating that the clock mechanism is also controlling limb outgrowth (Pascoal *et al.*, in preparation). It is therefore tempting to postulate that the rearrangement of cells into tissues and organs requires different coordinated events controlled by a common mechanism that counts time.

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