

Patterning embryos with oscillations: structure, function and dynamics of the vertebrate segmentation clock

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Summary

The segmentation clock is an oscillating genetic network thought to govern the rhythmic and sequential subdivision of the elongating body axis of the vertebrate embryo into somites: the precursors of the segmented vertebral column. Understanding how the rhythmic signal arises, how it achieves precision and how it patterns the embryo remain challenging issues. Recent work has provided evidence of how the period of the segmentation clock is regulated and how this affects the anatomy of the embryo. The ongoing development of real-time clock reporters and mathematical models promise novel insight into the dynamic behavior of the clock.

Key words: Gradient, Modeling, Negative feedback, Oscillator, Signaling, Somitogenesis

Introduction

The segmented anatomy of the vertebrate embryo is evident in the two bilaterally symmetrical rows of somites that flank the notochord along the body axis. These blocks of mesodermal cells give rise primarily to bone, muscle and skin of the adult body, which is correspondingly segmented. Somitogenesis is a rhythmic and sequential process in which each successive bilateral somite pair segregates at a regular time interval from the anterior end of the pre-somitic mesoderm (PSM, see Glossary, Box 1) as the body axis elongates (Fig. 1A,B). Somitogenesis has long been of interest to developmental biologists because it involves the coordination of patterning and growth of a tissue by a regularly repeated morphogenetic process. The topic of this review is the molecular segmentation clock that underlies this periodicity. The segmentation clock has attracted the attention of those interested in biological clocks and the molecular mechanisms of developmental timing, as well as those studying the function and stability of rapidly acting genetic circuits, and the interplay between the properties of single cells and their collective behavior at the tissue level. Finally, the rhythmic nature of the process is the seed for a theoretical interest in somitogenesis that is decades old and now promises a powerful synthesis of experiment and theory that is emblematic of modern embryology.

This review first provides an overview of somitogenesis, then describes the prevailing dynamic model for somitogenesis, the Clock and Wavefront mechanism, its molecular phenomenology

Box 1. Glossary

Biological oscillator. A system that generates a periodic variation in the state of a cell, tissue or organism. The vibrating stereocilia bundles of inner ear hair cells, the contraction cycle of cardiac muscle cells, circadian clocks and rhythmic neuronal circuits are all biological oscillators.

Coupling. Communication between neighboring oscillators that leads to mutual adjustment of their frequencies. For example, activation of Notch receptors in a presomitic mesoderm cell by Delta from neighboring cells can affect the dynamics of Notch pathway components in the target cell.

Frequency profile. Dependence of the frequency of the oscillators in an array on their position. In the segmentation clock, oscillators are proposed to be faster in the posterior and slower in the anterior, gradually slowing down across the tissue and so creating a spatial profile of frequencies.

Genetic oscillation. A type of biological oscillation in which regulation of gene expression generates oscillations in the levels of gene products.

Kinematic wave. A wave in which transport is absent or negligible. Examples include news ticker displays, in which the change of the state of the lamps, and not their movement, produces the moving pattern of words.

Negative feedback. An inhibitory control loop in which a downstream product inhibits an upstream step of a pathway, for example the inhibition of gene expression by the products of the gene.

Phase oscillator. A simplified representation of an oscillator where all physical variables, e.g. concentrations of oscillating species, are discarded and the dynamics are reduced to only one variable: the phase in the cycle. The phase is an abstract variable that measures the degree of progress of an oscillator through an oscillation cycle.

Pre-somitic mesoderm. The posterior-most and unsegmented paraxial mesoderm that will give rise to the somites and is directly continuous with the tailbud.

Segment. A repeated unit of subdivision in a tissue, typically along the anterior-posterior axis. A somite is a type of segment.

Somitogenesis period. The regular time interval between the formation of successive somite boundaries.

Steady state. A situation in which the dynamics of a given system does not change with time, as opposed to transient dynamics. An oscillator is in steady state when the change of its variables is identical in each cycle, implying that its period is constant.

Synchronization. Emergence of coherent dynamics among a population of oscillators. Noisy and incoherent oscillators can synchronize if the coupling between them overcomes noise and variability in individual oscillators.

Time delay. A time interval used to describe the completion of a process or series of processes that are not instantaneous, e.g. the time delay in producing an mRNA molecule is the time elapsed in its transcription and splicing.

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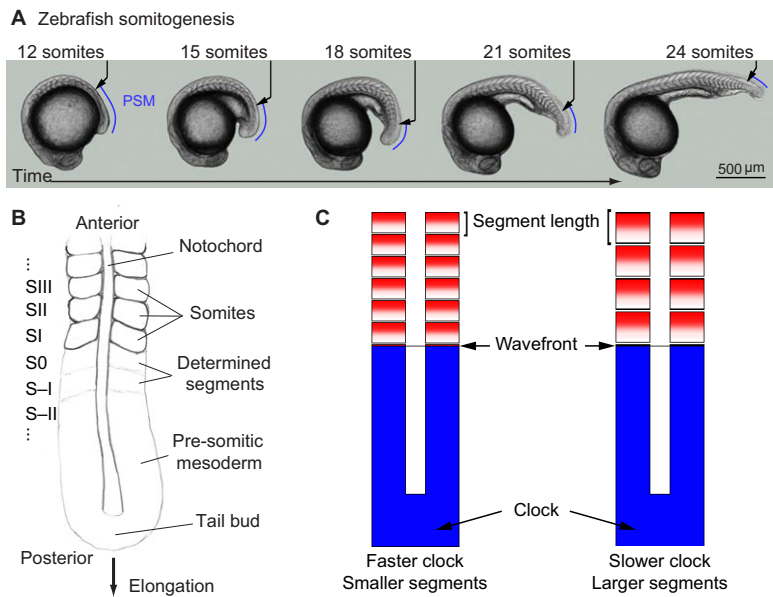


Fig. 1. Anatomy of the rhythmic and sequential segmentation process. (A) Advancing stages of zebrafish embryo somitogenesis, lateral view. Somites bud sequentially from the posterior unsegmented tissue; the pre-somitic mesoderm (PSM, blue line). The number of formed somites is listed and the position of the last formed somite is indicated with an arrow. Embryonic growth and the segmentation process are coordinated. (B) Dorsal view of the posterior of a segmenting vertebrate embryo. Somites form in pairs on each side of the notochord. Between the last formed pair of somites and the region of the PSM where cyclic gene expression occurs are determined segments that have not yet undergone epithelialization to become somites. The segments are labeled in the co-moving reference frame of the PSM according to Pourquié and Tam (Pourquié and Tam, 2001). (C) The Clock and Wavefront mechanism of vertebrate segmentation. The Clock is created by oscillating gene expression in the PSM (blue) and the Wavefront by a posteriorly moving front that arrests the oscillations of the Clock. Resulting segments (red/white) have a length that is determined by the period of the Clock multiplied by the regression velocity of the Wavefront (see also supplementary material Movies 1-3).

and components, and recent findings on the regulation of the period of the segmentation clock. We then organize what is known about the underlying molecular and cellular mechanisms of the segmentation clock into a three-tiered model, which begins with single-cell genetic oscillations (see Glossary, Box 1), moves on to the synchronization (see Glossary, Box 1) of these oscillators and ends with the controlled arrest of the oscillating cells. We also discuss the contributions of mathematical modeling to our understanding of this complex process and the many unanswered questions that remain.

An overview of somite development

Together with the tailbud, the PSM forms the posterior-most part of the vertebrate embryo. As the embryo elongates, driven by cell division and cell rearrangement, cells are added to the posterior of the PSM from the tailbud and are removed from the anterior into forming somites. It is often useful to describe this tissue in a co-moving reference frame in which the observer 'sits' atop the PSM and travels with it (Pourquié and Tam, 2001). In this perspective, cells appear to flow through the PSM. The PSM is thus a polarized tissue with immature mesodermal progenitors in the posterior transitioning to increasingly mature cells in the anterior. This polarity is set up by gradients of signaling factors that span the tissue, regulating the differentiation of the mesoderm and the position in the anterior where the somite boundaries will form (Aulehla and Pourquié, 2010). In most vertebrates, the PSM length gradually grows in early embryogenesis, then shrinks when somitogenesis removes more cells than are added (Gomez, et al., 2008).

Each somite is an internally polarized segment (see Glossary, Box 1), with the rostral and caudal halves having distinct properties. These half-segment identities are conferred through a set of genes that are expressed in the corresponding compartments before the morphological segment boundaries are visible (reviewed by Dahmann et al., 2011). The articulation of the spinal bones with the musculature that is crucial for locomotion is accomplished later in development by a process termed re-segmentation, in which the muscles attach to the bone derived from the neighboring half-segment ahead of the embryological origin of the muscle (Aoyama and Asamoto, 2000; Huang et al., 2000). The somite is also

polarized in the dorsal-ventral orientation, with skin deriving from the dorsal-most dermatome, muscle from the more central myotome and bone from the ventral sclerotome (reviewed by Christ and Scaal, 2008).

The segregation of each somite is accomplished by a morphogenetic rearrangement of a cohort of cells (reviewed by Dahmann et al., 2011). In most species, this forms a three-dimensional epithelial block that has a basal surface on the outside, which forms the somite boundaries, and a cluster of mesenchymal cells in the interior that contribute to the differentiated somite derivatives. The formation and stability of these morphological boundaries involves a mesenchymal-to-epithelial transition of the PSM tissue via coordinated changes in cellular shape and adhesion, and a deposition of extracellular matrix into the resulting gap or furrow (reviewed by Watanabe and Takahashi, 2010). These complex morphogenetic processes involve Eph-ephrin and integrin-fibronectin signaling, but lie outside of the scope of this review.

This review focuses on the mechanism by which the spatial pattern of somites is first generated. For this purpose, the key features of the somites are their anterior-posterior (AP) lengths and the temporal periodicity with which they are formed. Although both somite length and somitogenesis period (see Glossary, Box 1) change slowly and reproducibly along the AP axis during embryogenesis, these changes occur much more slowly than the rhythm of somitogenesis itself (Schröter et al., 2008). Thus it is both valid and useful to describe the fundamentals of somitogenesis in steady state (see Glossary, Box 1). Somite length and somitogenesis period also vary according to species: somitogenesis period is ~30 minutes in zebrafish, 90 minutes in chick and *Xenopus*, 2 hours in mouse, and 6 hours in human (Gomez et al., 2008). In externally developing species, the periodicity, like the overall rate of development, is temperature sensitive (Pearson and Elsdale, 1979; Schröter et al., 2008). Thus, somitogenesis appears broadly conserved at anatomical, morphogenetic and genetic levels, but when viewed in greater detail, every vertebrate segmentation clock performs its own elegant rhythmic dance. Before describing the molecular genetics of the segmentation clock that are responsible for these rhythms, we first discuss the unusual idea of using a biological oscillator (see Glossary, Box 1) to pattern an embryo.

The Clock and Wavefront mechanism

The rhythmic nature of somitogenesis suggests that a biological oscillator plays a role in this process. How is the rhythm of a biological oscillator used to segment an elongating body axis? Currently, the favored conceptual scheme for the regulation of somitogenesis is the Clock and Wavefront mechanism (Cooke and Zeeman, 1976), the fundamental components of which (Cooke, 1981) have significant experimental support. The Clock and Wavefront mechanism is a very general scheme that does not specify cellular or molecular details. The Clock consists of undefined **cellular oscillators** in the cells of the PSM that are coordinated to generate **coherent tissue-level oscillations**. In its simplest form, the **Wavefront moves posteriorly** across the PSM from the anterior, freezing the cellular oscillators as it passes and thereby leaving a permanent record of their activity at the time of arrest (Fig. 1C). In this way, the Clock and Wavefront mechanism translates the temporal information of an oscillator into a fixed periodic pattern in space (see supplementary material Movie 1). Regardless of the molecular components involved, the **length of each somitic segment (S)** can be mathematically described at steady state as being the **product of the velocity of the wavefront (v)** and the **period of the clock (T)**, $S=vT$ (see supplementary material Movies 1, 2).

During somitogenesis, **cells are continuously added** to the posterior of the PSM as **somites are formed from the anterior**, so the passage of the wavefront does not simply consume the PSM. In principle, segmentation could continue as long as the wavefront keeps moving, and posterior cells keep oscillating. Regardless of how segmentation is finally terminated, **at steady state the total number of segments (n)** will be given by the **total duration of segmentation (d)** divided by the **period of the clock (T)**, $n=d/T$. Thus, the hallmark of the Clock and Wavefront mechanism is that the period of the clock plays a causal role in determining the size and number of somites, and thus the anatomy of the vertebrate embryo. With this general dynamic model in hand, we now turn to the evidence for a biological oscillator at work during segmentation.

The molecular segmentation clock

A **molecular counterpart** to the morphological rhythm of somitogenesis, termed the segmentation clock (see Box 2), was first **discovered in the chick embryo** (Palmeirim et al., 1997) and

Box 2. A segmentation clock or an oscillating ruler?

The **circadian clock** is probably the best-understood biological clock (Hogeweg and Ueda, 2011). An important feature of the circadian clock, and indeed any clock, is **temperature compensation** of the period, which allows time to be measured accurately by the organism, regardless of short-term fluctuations in environmental conditions, such as the weather. By contrast, the **period of somitogenesis is not temperature compensated**. In those species that develop externally to the mother, the somitogenesis period becomes shorter at higher temperatures (Pearson and Elsdale, 1979; Schröter et al., 2008). Growth rate also increases with elevated temperature, and the period of somitogenesis exactly matches this increase so that the somite length is temperature compensated (Schröter et al., 2008). This preserves the proportions of the body plan, and thus its biomechanical properties. Thus, the **segmentation clock** that underlies somitogenesis is actually **a poor clock**, because its period changes dramatically with temperature, but it appears to be **a good ruler**, because the length of body segments and the final proportions of the body plan do not vary with temperature.

Box 3. Evolution of segmentation

Most metazoans are segmented along their anterior-posterior axis. Although some arthropods show **wave-like Notch pathway** gene expression patterns in their posterior growth zones (Chipman and Akam, 2008), definitive evidence for a segmentation clock in non-vertebrate species is still lacking (Damen, 2007). For example, ***Drosophila***, certainly the best-understood system, **does not use oscillations** to segment its body. How is the diversity of the observed vertebrate body forms generated and how is somitogenesis regulated in these different bodies (Richardson et al., 1998)? Transitions between segment numbers in evolution could be brought about by **alterations in the ratio of the period of the clock to the duration of somitogenesis** (Gomez et al., 2008); indeed, a **change in vertebral number** in a viable fertile zebrafish mutant that is due to a change in the period of the clock has been recently reported (Schröter and Oates, 2010). Oscillating signal transduction pathways are also conserved but **different individual genes are cyclic in different species** (Krol et al., 2011) (see also Table 1). For example, **Delta expression is cyclic in mouse and zebrafish but not in chick**, and **lunatic fringe expression is cyclic in mouse and chick but not in zebrafish**. Only **orthologs of *Hes1* and *Hes7*** are cyclic in **all species so far examined** (Krol et al., 2011), implying that these genes are part of an ancestral vertebrate segmentation clock mechanism.

Was the last common ancestor of bilateria segmented or did segmentation evolve independently in the extant lineages? As the Clock and Wavefront mechanism is generic, any oscillatory circuit could in principle provide the necessary activity for the Clock. **Transcriptional repressors** are the **basic building blocks** of genetic oscillators, and the genomes of metazoans are replete with these genes, suggesting that it might be common to evolve oscillatory circuits. Thus, what evidence would be necessary to demonstrate conclusively a shared common ancestry for a segmentation clock is not clear.

has since been observed in other vertebrate species, including the mouse, snake, frog and fish (Table 1). Here, we introduce the striking oscillating patterns of gene expression that reveal the molecular segmentation clock.

Traveling waves of cyclic gene expression

Oscillating patterns of gene expression are present in the **PSM and tailbud** of all examined **vertebrate** embryos, which indicates that these oscillations are an evolutionarily conserved mechanism (see Box 3). mRNA in situ hybridization experiments have defined a small group of so-called cyclic genes that display similar oscillating mRNA patterns (Fig. 2A, Table 1). Proteins encoded by some of these cyclic genes have oscillating levels, as shown by antibody staining (Bessho et al., 2003; Dale et al., 2003), indicating that unstable mRNA and proteins of cyclic genes are expressed and degraded in every cycle of the segmentation clock. Nevertheless, protein levels from very few cyclic genes have been measured, and it remains to be seen how many exhibit meaningful protein oscillations (Wright et al., 2009).

The key observable features of these oscillating patterns are: (1) a **traveling wave** of gene expression **moving anteriorly** through the PSM; (2) the slowing and **shortening of the wave in the AP direction**, and its **arrest in the anterior of the PSM** at a position that prefigures prospective **somite boundaries**; and (3) the repetition of the pattern with the formation of each new somite pair. These properties of the traveling wave patterns have been confirmed by time-lapse microscopy in living mouse embryos using luminescent

Table 1. Expression and genetics of cyclic (oscillating expression) segmentation clock genes

Name	Function	Species	Cyclic expression	Segmentation phenotype	Citations
<i>Hairy1</i>	Transcriptional repressor	Chick	mRNA in situ	Not determined	(Palmeirim et al., 1997)
<i>Lfng</i>	Notch glycosylase	Chick	mRNA in situ Western blot	Yes	(Aulehla and Johnson, 1999; Dale et al., 2003; McGrew et al., 1998)
<i>Hes7</i>	Transcriptional repressor	Mouse	mRNA in situ Protein (WMIC)	Yes	(Bessho et al., 2003; Bessho et al., 2001; Ferjentsik et al., 2009; Takashima et al., 2011)
<i>Hes1</i>	Transcriptional repressor	Mouse	YFP transgene mRNA in situ	No	(Masamizu et al., 2006; Ohtsuka et al., 1999)
<i>Hes5</i>	Transcriptional repressor	Mouse	Luciferase transgene mRNA in situ	No	(Masamizu et al., 2006; Ohtsuka et al., 1999)
<i>Dll1</i>	Notch ligand	Mouse	mRNA in situ	Yes	(Hrabe de Angelis et al., 1997; Maruhashi et al., 2005)
<i>Lfng</i>	Notch glycosylase	Mouse	mRNA in situ YFP transgene	Yes	(Aulehla and Johnson, 1999; Aulehla et al., 2008; Evrard et al., 1998; Forsberg et al., 1998; Zhang and Gridley, 1998)
<i>Nrarp</i>	Destabilizes Notch ICD	Mouse	mRNA in situ	Yes	(Kim et al., 2011; Wright et al., 2009)
<i>Dkk1</i>	Extracellular regulator of Wnt signaling	Mouse	mRNA in situ (intron?)	Yes	(Dequeant et al., 2006; MacDonald et al., 2004)
<i>Dact</i>	Intracellular Wnt signal transduction	Mouse	mRNA in situ	Yes*	(Suriben et al., 2006; Suriben et al., 2009)
<i>Axin2</i>	Cytoplasmic inhibitor of Wnt signaling	Mouse	mRNA in situ	No	(Aulehla et al., 2003; Yu et al., 2005)
<i>Snail</i>	Transcriptional repressor	Mouse	mRNA in situ	Yes*	(Carver et al., 2001; Dale et al., 2006)
<i>Dusp4</i>	ERK Phosphatase in FGF signaling	Mouse	mRNA in situ Western blot Protein (WMIC)	No	(Al-Mutairi et al., 2010; Niwa et al., 2007; Niwa et al., 2011)
<i>Lfng</i>	Notch glycosylase	Corn snake	mRNA in situ	Not determined	(Gomez et al., 2008)
<i>Esr9</i>	Transcriptional repressor	<i>Xenopus</i>	mRNA in situ	Not determined	(Li et al., 2003)
<i>her1</i>	Transcriptional repressor	Zebrafish	mRNA in situ	Yes	(Holley et al., 2000; Sawada et al., 2000)
<i>her7</i>	Transcriptional repressor	Zebrafish	mRNA in situ	Yes	(Henry et al., 2002; Oates and Ho, 2002)
<i>deltaC</i>	Notch ligand	Zebrafish	mRNA in situ Protein (WMIC)	Yes	(Jiang et al., 2000; Julich et al., 2005; Oates et al., 2005)
<i>her7</i>	Transcriptional repressor	Medaka	mRNA in situ	Not determined	(Elmasri et al., 2004)

The cyclic segmentation clock components referred to in this work are listed here with a brief overview of evidence for oscillating expression and a focus on loss-of-function phenotypes. In some cases, informative gain-of-function phenotypes are also cited. For a recent comparison and discussion of cyclic genes in different species see Krol et al. (Krol et al., 2011).

*Severe axial deformations in these embryos preclude assessment of the specificity of the segmentation defects.

Dact, dapper homolog, antagonist of β -catenin; Dkk, dikkopf homolog; Dll, delta like; Dusp, dual specificity phosphatase; Esr9, enhancer of split related 9; her, hairy related; Hes, hairy and enhancer of split; Lfng, lunatic fringe; Nrarp, Notch-regulated ankyrin repeat protein; WMIC, whole-mount immunohistochemistry.

and fluorescent transgenic reporters of oscillating segmentation clock genes (Fig. 2B, Table 1) (Aulehla et al., 2008; Masamizu et al., 2006; Takashima et al., 2011).

As these traveling waves of gene expression are the most obvious manifestation of the molecular segmentation clock, yet did not appear in the formulation of the Clock and Wavefront mechanism above, it is worth discussing their origin and implications. The waves of gene expression sweep across the field of PSM cells, indicating that they are not tied to a segment-specific cell lineage (Masamizu et al., 2006; Palmeirim et al., 1997). Furthermore, the maintenance of the oscillating gene expression patterns for some time after the PSM has been surgically transected or explanted indicates that these patterns do not require the bulk transport of material or the propagation of signals (Masamizu et al., 2006; Palmeirim et al., 1997). This implies that the patterns are the result of a wave of gene expression timing, also called a kinematic wave (see Glossary, Box 1). Individual cells in different positions along the AP axis of the PSM are in different phases of the

oscillating gene expression cycle. This AP phase profile manifests as a visible pattern of cyclic gene expression waves. Given a kinematic wave with the properties (1-3) described above, the observed phase profile implies a gradual slowing of the frequency of oscillators across the PSM (Palmeirim et al., 1997) (Fig. 2C, supplementary material Movie 4). Regardless of the underlying mechanism through which this frequency profile (see Glossary, Box 1) is established, alterations to the profile change the number and length of traveling waves within the PSM (Fig. 2C,D). The observed differences between species in the number of traveling waves of gene expression (e.g. three in zebrafish and one in mouse) imply differences in the frequency profile.

Importantly, the traveling wave pattern in the PSM repeats itself with every newly forming somite. This fact indicates that, regardless of the details of the waves or the arrest mechanism, the entire multicellular system of PSM and tailbud oscillates with a regular rhythm corresponding to the rate of somite formation. Thus, the molecular segmentation clock observed in vivo is consistent

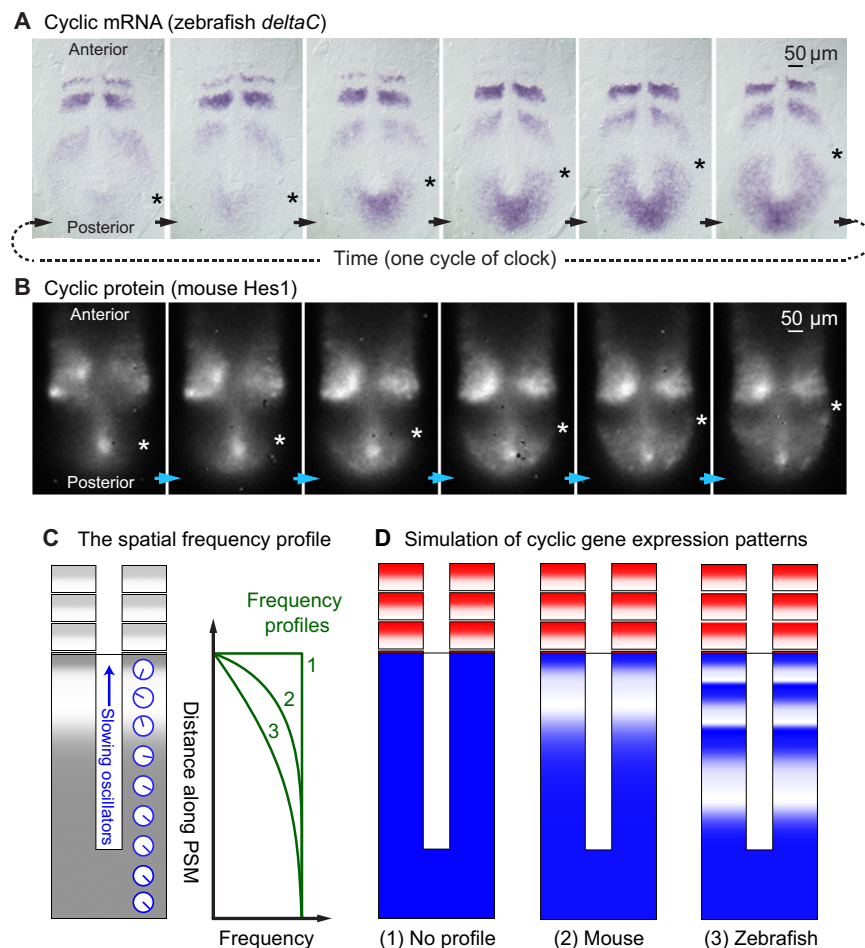


Fig. 2. Traveling waves of gene expression in the PSM. (A) In situ hybridization of *deltaC* mRNA in the pre-somitic mesoderm (PSM) of six individual six-somite zebrafish embryos (dorsal view). Embryos are staged sequentially, showing evolution of the gene expression pattern during one cycle of the segmentation clock. Waves of gene expression appear in the posterior PSM and move anteriorly (asterisk), becoming thinner as they do. Three traveling waves are seen in zebrafish at this developmental stage. (B) Bioluminescence imaging of the PSM of a mouse embryo at E10.5 expressing a *Hes1* promoter-driven luciferase reporter, showing a single traveling wave of expression (asterisk) from posterior to anterior. (C) Frequency profiles (green) of slowing oscillators (blue) across the PSM (gray). The phase of the oscillators is represented by the angle of the hand of a clock. The slowing of oscillators from posterior to anterior as they approach the arrest front is described by a spatial profile of their frequency. (D) Simulation of a model of the segmentation clock (Morelli et al., 2009), showing traveling waves of gene expression (blue) in three scenarios: sudden arrest (no profile), rapid slowing (mouse) and gradual slowing (zebrafish). The waves appear owing to the frequency profile of oscillating cells across the PSM. In profile 1, oscillators are stopped abruptly at the wavefront, which corresponds to the original proposal of the Clock and Wavefront mechanism (Cooke and Zeeman, 1976). In profiles 2 and 3, the oscillators slow down gradually with increasingly shallow frequency profiles, illustrating the typical traveling wave patterns found in mouse and zebrafish, respectively. Despite the differences in traveling waves, the simulations in all three panels have an identical segmentation period, wavefront velocity and resulting segment length (supplementary material Movie 4). (A) Reproduced, with permission, from Oates et al. (Oates et al., 2009). (B) Reproduced, with permission, from Masamizu et al. (Masamizu et al., 2006).

with the basic relationship that exists between segment length, wavefront velocity and clock period, as is predicted by the Clock and Wavefront mechanism. The correlation of cyclic gene expression patterns with somite formation suggests that the molecular segmentation clock may be causal in this process and we next examine the evidence that supports this idea.

Molecular genetics of the segmentation clock

The segmentation clock appears to be a complex oscillating genetic network (see also Box 4) (Dequeant et al., 2006; Krol et al., 2011). Members of this network include the cyclic genes, which encode a range of proteins that are primarily linked to three major signaling pathways: Delta-Notch, Wnt and fibroblast

growth factor (FGF). In addition, members of the Hes/Her (hairy and enhancer of split/hairy related) family of bHLH transcriptional repressors are cyclic in somitogenesis in all species examined (Krol et al., 2011) (Table 1). The role of most cyclic genes in the segmentation clock is not known, but here we attempt to categorize these cyclic genes based of the evidence from loss-of-function phenotypes.

Loss of function of several mouse and zebrafish cyclic genes yields specific somitogenesis phenotypes that are characterized by defective somite boundaries and disrupted traveling wave patterns (Table 1) (Bessho et al., 2001; Evrard et al., 1998; Henry et al., 2002; Hrabe de Angelis et al., 1997; Julich et al., 2005; MacDonald et al., 2004; Oates and Ho, 2002; Zhang and Gridley, 1998). In

Box 4. Starting and finishing segmentation

Although the material in the review focuses on the notion of steady-state segmentation, the **segmentation of a vertebrate embryo has a beginning and end**. Oscillation of the segmentation clock **begins during gastrulation**, long before the first somite forms (Ishimatsu et al., 2010; Jouve et al., 2002; Riedel-Kruse et al., 2007). The spatial patterns of the first oscillations are distinct from the later traveling waves, and the emergence of the waves may be tied to spatial **changes in fibroblast growth factor (FGF) signaling** that emerge as the **mesoderm elongates** (Ishimatsu et al., 2010). A function for these **'warm up' oscillations** remains to be established, but it appears to have no connection to the potential segmentation of the head mesoderm (Jouve et al., 2002).

How segmentation ceases is both an embryological and an evolutionary question, as changing when segmentation stops could lead to changes in segment number and body proportions. What cues determine when segmentation stops? The period of somitogenesis lengthens in the tail (Schröter et al., 2008). Cyclic gene expression changes as the **tailbud and presomitic mesoderm (PSM) shrink in size at the end of somitogenesis**, and the clock stops even though the PSM is still present (Tenin et al., 2010). One recent hypothesis to explain the cessation of the segmentation clock is that as the PSM becomes smaller, the level of **somite-derived retinoic acid** rises in the tail bud, eventually repressing the high level FGF signaling required for ongoing oscillations (Gomez et al., 2008; Tenin et al., 2010); however, functional evidence for this is lacking. Alternatively, it is possible that the embryo has a **separate timing mechanism** to shut off the segmentation clock in coordination with the end of elongation.

some **mutants of cyclic genes** from the FGF and Wnt pathways, such as **mouse *Snail***, which encodes a **FGF-inducible transcriptional repressor**, and ***Dact1***, which encodes a Wnt pathway signal transduction component, axial deformations and truncations potentially mask the specific functions of these genes in segmentation (Carver et al., 2001; Suriben et al., 2009). Some cyclic gene mutants, such as the mouse bHLH transcriptional repressors ***Hes1*** and ***Hes5***, and the Wnt-pathway signal transduction component ***Axin2***, have no observable segmentation phenotype (Ohtsuka et al., 1999; Yu et al., 2005). For others, the severity of the phenotype is increased in double knock-down combinations, for example zebrafish bHLH transcriptional repressors ***her1;her7*** and ***her1;hes6*** (Henry et al., 2002; Oates and Ho, 2002; Sieger et al., 2006). This indicates that the architecture of the segmentation clock is partially redundant, and its function is robust to perturbation.

Many genes from these pathways are expressed in the PSM but without a traveling wave pattern, and mutations in some of these genes, such as those encoding the cell-cell signaling receptor ***Notch1***, its ligand Delta-like 3 and the activated Notch protease presenilin, also disrupt segmentation (Conlon et al., 1995; Dunwoodie et al., 2002; Shen et al., 1997; Wong et al., 1997) (Table 2). In some cases, the **encoded protein is modified** or **localized in a periodic manner**. For example, the cleaved Notch intracellular domain, which is indicative of active Notch signaling, is visible in a traveling wave pattern in mouse PSM (Huppert et al., 2005; Morimoto et al., 2005). From enhancer dissection studies of the mouse cyclic lunatic fringe (***Lfng***) gene, which activates Notch signaling in the PSM via glycosyltransferase activity, it has been proposed that Notch signaling is primarily required in the anterior PSM downstream of the segmentation clock (Shifley et al., 2008; Stauber et al., 2009). However, the existence of weak residual posterior PSM oscillations driven by the tested enhancer constructs

(Stauber et al., 2009) and the ability of the posterior-specific cyclic regulatory region of ***Hes7*** to rescue completely the loss of function ***Lfng*** phenotype (Oginuma et al., 2010) suggest that **oscillating Notch signaling** in the segmentation clock itself is essential for mouse somitogenesis.

Another example of post-translational oscillation is the periodic phosphorylation in the mouse PSM of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) serine/threonine kinase, a key component of FGF signal transduction (Niwa et al., 2011). Cyclic expression in the PSM of the ERK phosphatase dual specificity phosphatase 4 (***Dusp4***), itself an FGF target gene, may be responsible for the observed rhythm in ERK phosphorylation (Niwa et al., 2007; Niwa et al., 2011). Interestingly, **sustained oscillations in the ERK signaling network** have been observed in other contexts (Shankaran and Wiley, 2010), but segmental defects have not been reported in ***Dusp4*** mutant mice (Al-Mutairi et al., 2010), and the role of these intriguing phosphorylation cycles in the mouse segmentation clock remains unclear.

In combination, these results indicate that cyclic genes are the **transcriptionally oscillating component** of a larger molecular segmentation clock. Whenever cyclic traveling wave patterns are disrupted, normal somitogenesis is also disrupted. Thus, these phenotypes are consistent with the idea that **cyclic genes** and their **linked signaling pathways** function in a molecular segmentation clock that regulates somitogenesis.

Although these mutations identify some of the components of the segmentation clock, their disruption does not address the role of timing in somite patterning. To determine whether the embryo uses a Clock and Wavefront mechanism to time the regular and sequential formation of somites, mutations or other perturbations that change the period of the clock but do not concomitantly disrupt somite boundaries or axial extension need to be investigated.

Regulation of period in the molecular segmentation clock

Recent experimental and theoretical advances have explored the **control of the period of the clock**, as well as how the embryo uses this timing to control the size, number and identity of the resulting segmented structures. Through the use of a sensitive and precise multiple-embryo time-lapse protocol that measures the period of somitogenesis in freely developing zebrafish embryos, a series of mutations in genes that encode components of the Delta-Notch signaling pathway were recently discovered to alter the period of somitogenesis (Herrgen et al., 2010; Schröter and Oates, 2010). These mutations cause **posterior segmentation defects**, yet leave the **somite boundaries of the anterior trunk largely intact** (Holley et al., 2000; Holley et al., 2002; Itoh et al., 2003; Julich et al., 2005). Although the mutant embryos **extend normally with a wild-type wavefront velocity**, these **anterior somites form with a longer period** and have a correspondingly increased length (Herrgen et al., 2010). This observation is consistent with the first expectation of the Clock and Wavefront mechanism, that a slower clock will cause longer segments, $S=vT$. A longer segmentation clock period is also predicted to change the length of the traveling waves of gene expression in the PSM (Morelli et al., 2009), a prediction that was confirmed by measurements of this length distribution in mutant embryos (Herrgen et al., 2010). Thus, the changes in Delta-Notch mutant embryos are consistent with a change in the period of the molecular segmentation clock.

A mutation in the gene that encodes the ***Hes6*** bHLH transcriptional repressor protein gives rise to a highly informative period phenotype. ***hes6*** mutant zebrafish embryos extend their axis

Table 2. Expression and genetics of non-cyclic segmentation clock genes

Name	Function	Species	Oscillatory activity	Segmentation phenotype	Citations
<i>Notch1</i>	Delta receptor	Mouse	Proteolytic cleavage of intracellular domain	Yes	(Conlon et al., 1995; Huppert et al., 2005; Morimoto et al., 2005)
<i>Dll3</i>	Notch ligand	Mouse	ND	Yes	(Dunwoodie et al., 2002)
<i>Psen1</i>	Gamma secretase activity, cleaves NICD	Mouse	ND	Yes	(Shen et al., 1997; Wong et al., 1997)
<i>Mapk1</i>	A kinase involved in FGF signaling	Mouse	ERK2 phosphorylation	No*	(Niwa et al., 2011; Yao et al., 2003)
<i>hes6</i>	Transcriptional repressor	Zebrafish	ND	Yes	(Schröter and Oates, 2010)
<i>deltaD</i>	Notch ligand	Zebrafish	ND	Yes	(Herrgen et al., 2010; Holley et al., 2000)
<i>mindbomb</i>	Delta ubiquitin ligase	Zebrafish	ND	Yes	(Herrgen et al., 2010; Itoh et al., 2003)
<i>notch1a</i>	Delta receptor	Zebrafish	ND	Yes	(Herrgen et al., 2010; Holley et al., 2002)

The non-cyclic segmentation clock components referred to in this work are listed here with a brief overview of evidence for oscillating activity and a focus on loss-of-function phenotypes.

*Mutant mouse does not form mesoderm, precluding assessment of a specific role in segmentation.

Dll3, delta-like 3; *hes6*, hairy and enhancer of split 6; *Mapk1*, mitogen-activated protein kinase 1; *Psen1*, presenilin 1.

normally with a wild-type wavefront velocity, but their somites form more slowly and are correspondingly longer, again as would be predicted by the Clock and Wavefront mechanism (Schröter and Oates, 2010). Furthermore, because *hes6* mutants can complete segmentation without boundary defects in the same total time as wild-type siblings do, it is possible to determine how many segments they form altogether. The striking result is a reduction in total segment number that is in quantitative agreement with the slowing of the period of somitogenesis. This is consistent with the second expectation of the Clock and Wavefront mechanism, $n=d/T$.

Other recent papers have examined the regulation of the period of the segmentation clock in chick and mouse. Inhibition of the canonical Wnt pathway results in slower somite formation and causes alterations to cyclic gene expression patterns in chick and mouse embryos, although somite length was not reported to be longer, as would be expected according to the Clock and Wavefront mechanism (Gibb et al., 2009). This apparent contradiction may be explained by concomitant alterations in the velocity of the wavefront, perhaps owing to changes in embryonic extension (Wilson et al., 2009). A role for Wnt signaling in regulating the period of the segmentation clock has been previously discussed (Aulehla et al., 2003; Aulehla et al., 2008), and this new work will spur further investigation.

Surgical separation of the notochord from the paraxial mesoderm in chick results in a dramatic slowing of somite formation accompanied by altered cyclic gene expression patterns on the separated side (Resende et al., 2010). Inhibition of sonic hedgehog (Shh) signaling mimics these phenotypes and addition of exogenous Shh can rescue the effect, as can exogenous retinoic acid (RA), implicating these pathways in the timing of somite formation in chick. The Shh pathway had not previously been implicated in the segmentation clock, and how it might integrate with the known clock pathways remains to be determined. As in the study mentioned above (Gibb et al., 2009), changes to somite length were not reported. Without assessment of segment length or wavefront velocity in these treatments, the implications for a Clock and Wavefront mechanism in chick are unclear.

Investigation of somitogenesis in mice with a mutation in the Notch target gene Notch-regulated ankyrin repeat protein (*Nrarp*) reveals a slight lengthening of the somitogenesis period and a corresponding decrease in somite and vertebral body number (Kim et al., 2011). *Nrarp* encodes a negative regulator of Notch signaling,

and molecular markers of Notch signaling are upregulated in the *Nrarp* mutant PSM. Strikingly, pharmacological inhibition of Notch signaling results in an increase in somite number over wild type, suggesting a reduced somitogenesis period. Without measurements of wavefront velocity, somite length or total duration of somitogenesis, these data are inconclusive with regards to a Clock and Wavefront mechanism, but are nevertheless consistent with a role for Notch signaling in regulating the period of the segmentation clock in mouse. Why does loss of Notch signaling increase the period of somitogenesis in zebrafish (Herrgen et al., 2010), but decrease it in mouse (Kim et al., 2011)? The answer is not known, but this difference between zebrafish and mouse was previously predicted to be due to different relative delays in the coupling between oscillating cells (Morelli et al., 2009).

Combined, these recent discoveries identify genes that appear to control the period of the segmentation clock and also describe more generally how this temporal period is translated into the periodic segmentation of the vertebrate anatomy (see also Box 5). Although direct imaging of the molecular segmentation clock in somitogenesis period mutants has yet to be reported, the mutant studies described above are consistent with a Clock and Wavefront mechanism controlling vertebrate segmentation. However, testing the Clock and Wavefront mechanism is fraught with experimental and conceptual difficulties. In general, testing requires some measurement or estimation of period, wavefront velocity and segment length in the same experiment. Theoretical studies suggest that alterations to the length of the PSM or in the frequency profile may cause changes in cyclic gene expression patterns independently of the period of the segmentation clock (Giudicelli et al., 2007; Gomez et al., 2008; Morelli et al., 2009). Thus, care must be taken when equating altered cyclic expression patterns with altered segmentation clock period. Furthermore, measurement of period must be carried out with a sampling interval that is sufficiently shorter than the period, in order to allow the detection of small changes or longer-term trends in the period. Finally, care must be taken to distinguish between somitogenesis period and the period of the underlying molecular segmentation clock; these do not have to be the same. Use of theory can guide the measurement and interpretation of static images (Giudicelli et al., 2007; Herrgen et al., 2010), but integrating imaging of live reporters of the segmentation clock will play a crucial role in future studies (Aulehla et al., 2008; Masamizu et al., 2006; Takashima et al., 2011).

Box 5. Can the embryo count segments?

How does the embryo make the correct number of segments with the correct anterior-posterior identities of the body plan? There are two basic hypotheses for the observed very tight regulation of segment number and identity. First, the embryo could 'count' the number of oscillations and finally stop segmenting when the correct number was reached. AP identities would be assigned according to the increasing number (Dubrulle et al., 2001; Jouve et al., 2002). In this scheme, the period of segmentation would not directly impact either segment number or AP identity. Alternatively, the embryo might allocate a defined time window during development for segmenting, which in combination with a tight control of the period of the segmentation clock would yield the same number of segments every time (Cooke and Zeeman, 1976). Segmental AP identities would be instructed by some independent form of positional information along the axis. In this scheme, segment number and identity would be affected by changes to the period of segmentation.

The zebrafish *hes6* mutant has a slower somitogenesis period and makes fewer longer segments in the same total time as wild-type siblings (Schröter and Oates, 2010), in agreement with the latter hypothesis. Furthermore, in this normally elongating mutant, anatomical landmarks of AP regional identity are not positioned at the same segment number as in wild type, but rather shift segment number to remain in the same absolute position in the body plan. From this, it would appear that vertebrate embryos do not count their segments, either to determine their total number or their regional identity. The segmentation clock thus appears to be acting in parallel to some higher-level control of body extension and regional identity.

The picture of the molecular segmentation clock that emerges from the different experiments in various species appears somewhat fragmented and, in places, apparently contradictory. We will argue below that it is nevertheless possible to place these findings within a framework that allows general properties to be highlighted and so make fertile avenues of inquiry apparent.

A three-tier model of the segmentation clock

In order to synthesize the Clock and Wavefront mechanism with experimental data on the molecular segmentation clock and with mathematical models of segmentation, we outline below a preliminary framework for organizing this information, called the three-tier model, in which the clock is defined as a multi-scale rhythmic pattern generator whose output is at the tissue level (Fig. 3). Although each of the tiers in this model corresponds to distinct experimental and theoretical investigations, the processes within each tier must integrate seamlessly within the embryo for the segmentation clock to function.

In the bottom tier is the single cell oscillator (Fig. 3A). This is the idea that each of the cells in the PSM is capable of oscillating, at least for a time, in isolation. The genetic circuits that underlie these cell-autonomous oscillations are therefore central to our understanding of the period of the clock, and regulation of the period by various factors at other levels must at some point interact with these intrinsic pace-making circuits.

The middle tier is the local synchronization of oscillating cells (Fig. 3B). This is the idea that the observed coherent oscillation of many PSM cells requires an active synchronization mechanism. Synchronization implies that individual cells can measure the phase of their neighbors, and speed up or slow down accordingly to match them. Thus, the genetic circuits involved are cell-cell communication pathways that must interact with the cell-autonomous pace-making circuits.

The upper tier is the global control of the arrest of the oscillating cells (Fig. 3C). This is the idea that the position in the anterior PSM where the oscillating cells convert their temporal phase information into a stable periodic spatial pattern must be precisely regulated. The traveling waves of gene expression emerge in this tier. Control of positional information over hundreds of micrometers involves gradients of signaling molecules, and these signals must be able to regulate the pace-making circuit of the oscillators by some means.

We believe that this three-tier model provides a useful and explicit framework with which we can organize the diverse pieces of information about the segmentation clock. New data can be interpreted with respect to the tiers, experiments can be designed to test a tier, and if a new tier of description is discovered, it can be integrated into the framework. It is possible that the importance of a given tier will vary depending on the species. Of course, our organization of data and models within these categories does not itself explain the various molecular or dynamic mechanisms at work. Below, we discuss the evidence for these tiers and how they might interact. Mathematical models that have been influential in motivating experiments or tested against data are described. Unanswered questions are framed and listed in Box 6.

Single cell oscillations

What is the smallest oscillating unit in the PSM? Could it be an individual PSM cell, or do oscillations require intercellular communication, as is the case in reaction-diffusion models (Meinhardt, 1982)? PSM tissue dissected from the embryo exhibits traveling waves of endogenous cyclic genes, indicating that the information for the oscillations is contained within the PSM (Palmeirim et al., 1997). When chick PSM is further dissected into small pieces along the AP axis, each piece continues to oscillate approximately on schedule (Maroto et al., 2005). When single cells are dissociated from PSM and fixed in a time-series, variable expression of endogenous cyclic genes is observed, consistent with persistent oscillations in single cells (Maroto et al., 2005).

The most direct evidence for single-cell oscillations comes from time-lapse imaging of mouse cells that express a luciferase reporter driven by the *Hes1* cyclic gene promoter (Masamizu et al., 2006). Cultured fibroblasts carrying this transgene show a range of expression dynamics: some dampened the bioluminescent signal quickly, whereas others displayed persistent periodic fluctuations in luminescence over 12 hours. PSM cells isolated from a transgenic mouse carrying the same reporter showed a similar activity. The period and amplitude of these oscillations was variable, with an average period longer than expected from the intact mouse embryo. The noise of the individual oscillating cells appears much higher than the tissue-level variability (Masamizu et al., 2006; Riedel-Kruse et al., 2007; Schröter et al., 2008).

Thus, there is significant evidence to support the existence of single cell oscillators that underlie the rhythm of the segmentation clock. How are these oscillations generated at the molecular level?

Hes/Her auto-regulatory loop: a candidate pace-making circuit

From the list of known cyclic genes (Table 1), candidates for the generation of such single cell oscillations are the Hes/Her bHLH transcriptional repressors, as a transcription factor able to repress its own expression could build the simplest genetic auto-regulatory negative-feedback loop (Novak and Tyson, 2008) (Fig. 4). The mutation or knockdown of cyclic *Hes/Her* genes in

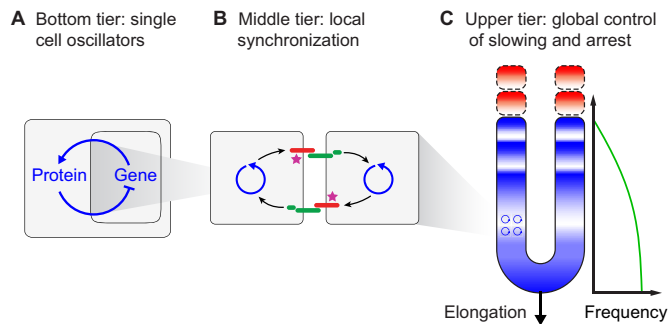


Fig. 3. Three-tier model of the segmentation clock. (A) Bottom tier: genetic oscillations produced by single-cell oscillators driven by delayed negative feedback. (B) Middle tier: intercellular coupling mediates local synchronization between neighboring cells. Blue circular arrows illustrate single cell oscillators, connected through black arrows to the signaling pathway (red ligands, green receptors). Stars indicate ligand trafficking processes. (C) Upper tier: tissue level cyclic gene expression patterns (blue/white) controlled by long-length scale gradients across the pre-somitic mesoderm (PSM), which slow down and arrest the oscillations (green frequency profile as described in Fig. 2C). Arrested segments are red/white. Small circles with arrows represent single cell oscillators. The arrow indicates the direction of embryonic elongation.

mouse and zebrafish leads to defective somite formation and to the disruption of cyclic gene expression, indicating that they are essential parts of the segmentation clock (Bessho et al., 2001; Henry et al., 2002; Oates and Ho, 2002; Sieger et al., 2006). The persistent oscillation of several Wnt and FGF pathway genes in the *Hes7*-null mutant mouse suggests that sub-circuits of the segmentation clock network are still active (Ferjentsik et al., 2009; Hirata et al., 2004), but the profound segmentation defects observed in these embryos indicate that this remaining activity is insufficient to pattern the PSM.

A role for Hes/Her-mediated negative feedback (see Glossary, Box 1) at the single-cell level is supported by observations and perturbations carried out *in vitro* and *in vivo*. Mouse *Hes1* mRNA and protein abundance oscillates in a variety of cell lines with a period approximately equal to mouse somitogenesis (Hirata et al., 2002). Biochemical data from manipulating *Hes1* production, half-life and activity in these cells indicate that unstable *Hes1* protein periodically represses its own expression. This regulatory logic was corroborated *in vivo* for *Hes7* by similar manipulations in wild-type embryos and in *Hes7* mutant mouse embryos, in which segmentation is severely defective (Bessho et al., 2003). Importantly, *Hes7*-binding N-box regulatory elements are found in the *Hes7* promoter, and *Hes7* protein is detected on its own promoter *in vivo* by chromatin immunoprecipitation, indicating that the auto-regulatory action is direct (Bessho et al., 2003; Chen et al., 2005).

Models of transcriptional oscillations (Lewis, 2003; Monk, 2003) require a negative-feedback loop, a time delay (see Glossary, Box 1) in the feedback, sufficient non-linearity and a balancing of the timescales of the reactions involved (Novak and Tyson, 2008). When oscillating, the period of the single-cell oscillator is set by delays that arise from a combination of the steps that make up one cycle: transcription and translation, the transport of molecules in and out of the nucleus, additional biochemical events, such as splicing and post-translational modifications, and the decay of the products (Fig. 4). Elegant studies have changed the stability of *Hes7* protein or the delays

Box 6. Questions for future research

Oscillation

At which steps in the negative-feedback loop can the period be regulated?

Can a mutant segmentation clock with an altered period be predictably engineered *in vivo*?

Can culture conditions be established that will allow reliable analysis of perturbations to individual oscillating cells?

Are there multiple parallel Hes/Her feedback circuits and, if so, what properties do these confer to the segmentation clock?

Do additional properties of the pre-somitic mesoderm (PSM) oscillate, perhaps in relation to ionic, metabolic or phosphorylation cycles?

Synchronization

How do PSM cells in embryos other than zebrafish stay synchronized? Are oscillations of fibroblast growth factor (FGF) and Wnt signaling in amniotes involved?

Are oscillating cells noisier and slower in Delta-Notch mutants than in a wild-type zebrafish embryo, as predicted?

Can coupling strength be measured directly by observing the rate of change in the phase of cells as they synchronize?

Do neighboring cells send smooth coupling signals of equal strength and timing, or is coupling effected with asymmetrical or discontinuous signaling?

Does elevated cell movement in the tailbud promote synchronization of the segmentation clock (Uriu et al., 2010)?

Arrest

What are the quantitative characteristics of the FGF, Wnt and retinoic acid gradients that span the PSM?

Why do transient signaling perturbations cause changes to segment length only after a defined delay? What does this reveal about the arrest mechanism?

Do individual oscillators slow across the PSM, as predicted by tissue-level patterns?

How do the signaling systems spanning the PSM act on the pace-making circuits of individual cells to arrest/sustain their oscillation? Does the topology or components of the pace-making circuit vary with position along the PSM or developmental stage?

Is each newly forming segment specified in its entirety or is the segmental pattern laid down gradually cell by cell?

associated with transcribing the *Hes7* gene, and these alterations disrupt oscillations *in vivo* in agreement with modeling predictions (Hirata et al., 2004; Takashima et al., 2011). However, there are potentially many ways to disrupt an oscillator, and disruption is not a definitive outcome for testing theories of an oscillating circuit (Oswald and Oates, 2011). Although the *Hes6* mutant appears to have an altered segmentation clock period (Schröter and Oates, 2010), directly connecting this gene family to control of the period, there are currently no models that explain this change.

Thus, although many questions remain unanswered (see Box 6), it seems likely that individual PSM cells possess a noisy single-cell oscillatory circuit that depends on the auto-regulatory activity of *Hes/Her* genes. In the next section, we discuss the transition from single cells to the multicellular organization of the segmentation clock.

Synchronization of neighboring oscillating cells

Depending on developmental stage and species, a forming somite can span tens to hundreds of cells. For these cells to be coordinated, gene expression must be coherent across a similarly sized domain. How can gene expression from many noisy,

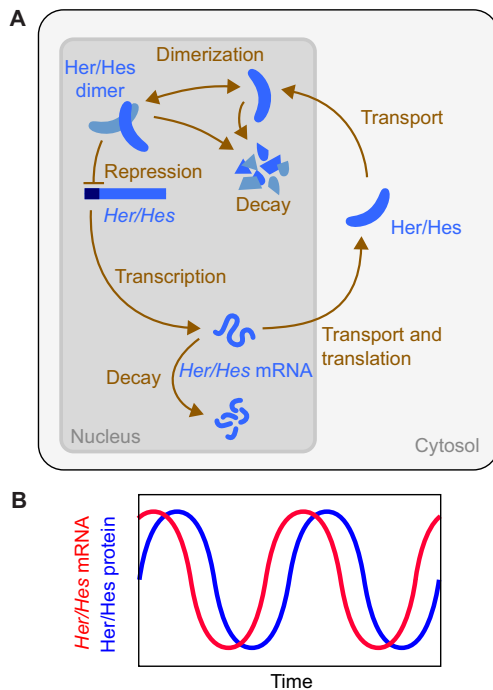


Fig. 4. Single cell genetic oscillators. (A) Schematic of a single-cell oscillator in which gene products repress gene activity. This negative-feedback loop involves a sequence of steps. The *Her/Hes* gene is transcribed with a basal rate and mRNA is transported from the nucleus and translated. Proteins are transported back into the nucleus where they dimerize to form a transcriptional repressor. This repressor accumulates and binds the gene promoter to inhibit gene expression. Finally, when gene products (mRNA, protein, dimer) decay, the gene repression is released and the cycle can start over again. (B) Oscillations of mRNA and protein concentrations in the single cell oscillator.

fluctuating individual oscillators be so well coordinated? How is the observed precision in the timing of somitogenesis achieved if the cellular timekeepers are so noisy, as described in the previous section? The answer is not unique to the PSM: noisy oscillators can be synchronized by coupling (see Glossary, Box 1) (Kuramoto, 1984; Winfree, 1967).

Delta-Notch signaling synchronizes neighboring PSM cells

Candidates for a local cell coupling mechanism can be found among the list of cyclic genes (Table 1) (Jiang et al., 2000). The Delta-Notch signaling system is a well-studied cell-cell communication pathway, with many functions in development and disease (Lai, 2004). Traveling waves of Notch pathway gene expression and Notch receptor activation (Huppert et al., 2005; Maroto et al., 2005) suggest that PSM cells signal to each other with every cycle of the clock (Fig. 5).

As mentioned above, mutations in zebrafish Delta-Notch signaling components cause posterior segmentation defects. The transition from normal to disrupted boundaries is sharp and reliably positioned along the AP axis at a mutant-specific somite number (Holley et al., 2000; Holley et al., 2002; Itoh et al., 2003; Julich et al., 2005). Underlying this morphological phenotype, the traveling waves of gene expression in the PSM slowly lose their coherence, decaying to a ‘salt and pepper’ uncorrelated pattern of juxtaposed high- and low-expressing cells (Jiang et al., 2000; Oates and Ho, 2002). This loss of coherence can be quantitatively compared with wild-type patterns using spatial autocorrelation functions that

measure the organization in the traveling wave patterns (Herrgen et al., 2010). Together, these data suggest that Delta-Notch signaling is a coupling mechanism that keeps the oscillations of the PSM cells locally synchronized (Jiang et al., 2000) (supplementary material Movies 5, 6).

Cyclic gene expression initiates simultaneously throughout the future mesoderm at the onset of gastrulation in zebrafish, even in the absence of Notch signaling (Riedel-Kruse et al., 2007). The transition point from ordered to disordered segments can be positioned along the axis by experimentally manipulating when Notch signaling is inhibited, as well as the level of Notch inhibition (Mara et al., 2007; Ozbudak and Lewis, 2008; Riedel-Kruse et al., 2007). After re-activating Delta-Notch coupling in the fully disrupted state, the PSM resynchronizes, coherent traveling waves are formed and normal segments are made once again. In combination with a theoretical description of the clock as a population of coupled phase oscillators (see Glossary, Box 1) (Kuramoto, 1984), these manipulations and observations can be related to the loss of synchrony in the clock (Riedel-Kruse et al., 2007). Using this theory, the time elapsed from Notch inhibition to the loss of synchrony in the PSM can be related to the noise level in the oscillations of individual cells and the strength of the coupling that tries to keep them synchronized.

The main function of PSM cell coupling is to prevent their desynchronization and the subsequent loss of a coherent genetic segmental pre-pattern. However, cells require time to make and deploy the large proteins of the Delta-Notch system, and this delay in coupling appears to introduce an additional dynamic effect in the period of the synchronized population of cells (Morelli et al., 2009). As mentioned above, when Delta-Notch signaling is compromised in zebrafish, somitogenesis period, segment length and the traveling wave pattern of cyclic gene expression show a coordinated change that is consistent with a change in the period of the segmentation clock (Herrgen et al., 2010). Furthermore, by gradually altering Notch signaling strength, the somitogenesis period can be gradually tuned. Thus, even though each cell has its own rhythm, the period of the segmentation clock of the embryo appears to be a fundamentally multicellular property, self-organized from the dynamic interactions between synchronized PSM cells. Whether delays in the coupling cause the population to speed up, as is observed in zebrafish, or slow down depends on the ratio of the delay to the period of oscillations (Morelli et al., 2009). Because of the longer period in mouse, it was predicted that the ratio of coupling to period in this species might cause a coupling-mediated slowing of the oscillations (Morelli et al., 2009), which has recently been experimentally reported (Kim et al., 2011).

How does coupling work in molecular terms? How does Notch signaling change the period of the single-cell pacemaker circuit? Zebrafish *Her* genes are transcriptional targets of Notch signaling, although not dependent on Notch, and local overexpression of *Delta* alters cyclic *Her* gene expression in neighboring cells (Horikawa et al., 2006). This local phase shift can be reproduced using a mathematical model of the *Her* feedback loop (Lewis, 2003) that describes a chain of cells coupled through Delta-Notch signaling (Horikawa et al., 2006). In addition to becoming desynchronized, the level of *Her* gene expression gradually decreases in the absence of Delta-Notch coupling (Oates and Ho, 2002; Oates et al., 2005; Ozbudak and Lewis, 2008), as might be expected from the loss of a weak positive transcriptional regulatory input. In turn, gain- or loss-of-function of *Her* genes perturbs *Delta* expression (Oates and Ho, 2002; Shankaran et al., 2007; Sieger et al., 2006). The current hypothesis is that, during a cycle of the

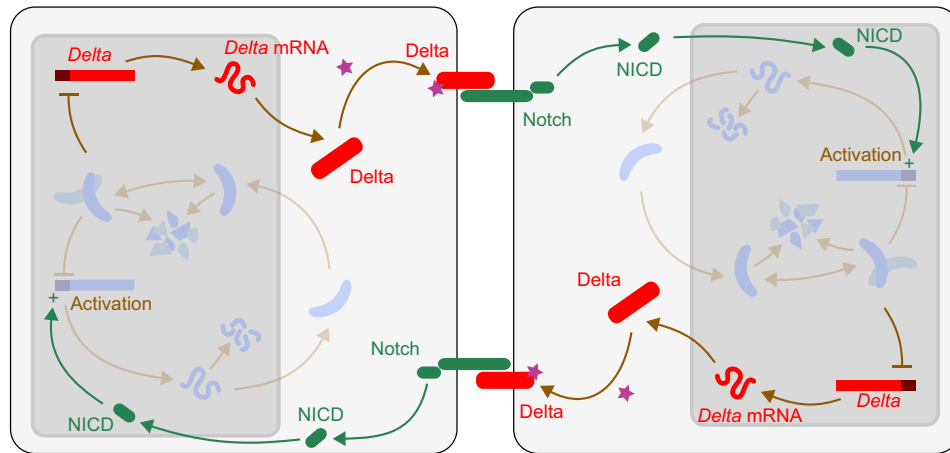


Fig. 5. Local synchronization through intercellular communication in the segmentation clock. How neighboring oscillating cells send and receive signals via the Delta-Notch pathway. The genes that encode the Delta ligands are repressed by the same factors that control the single-cell oscillator feedback loop, as shown in Fig. 4. The *Delta* gene is transcribed and the mRNA exported to the cytoplasm. The mRNA is translated, and the protein undergoes post-translational modifications (star) before becoming an active ligand for Notch receptors on the neighboring cell. Upon ligand/receptor binding, the Notch intracellular domain (NICD) is proteolytically cleaved from the Notch receptor of the neighboring cell and translocates to the nucleus, where it positively regulates the transcription of cyclic genes, such as the *Hes/Her* gene. Notch signaling is not required for basal *Hes/Her* gene transcription. The details of the feedback loop of single-cell oscillator (Fig. 4) are dimmed to highlight coupling in this figure. Pre-somitic mesoderm cells communicate with multiple neighbors, effectively sitting in a 3D lattice of mobile coupled oscillators.

segmentation clock, the timing of the Notch signal changes the timing of transcriptional initiation of *Her* genes in the receiving cell, and, reciprocally, this change is communicated to neighboring cells by the change in timing of *Delta* gene repression (Fig. 5). Combined, these changes in timing comprise the regulation of the period of the cells via coupling.

Thus, although it seems clear that cell-cell communication brings coherence to the oscillators of the PSM, unanswered questions about the synchronization of neighboring oscillating cells remain (see Box 6). In the next section, we discuss the slowing and arrest of oscillations at the tissue level.

Arresting cell oscillations: global control

Somite progenitor cells have access to several gradients of positional information across the PSM that arise from release and propagation of signaling molecules from the somites and tailbud (Fig. 6). How do the PSM cells read these gradients to regulate their oscillations? In the Clock and Wavefront mechanism, the position in the PSM where cells arrest is of fundamental importance, because it is the movement of this point with the elongating tissue that corresponds to the velocity of the wavefront. Following the relationship $S=vT$, alterations to wavefront velocity v should cause changes in segment length S .

In all cases, oscillating gene expression arrests in the anterior PSM. Although the expression of some cyclic genes persists in formed segments, it does not oscillate (Jiang et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997). The position where the oscillations arrest can be inferred by fitting the traveling wave patterns of cyclic genes to models of the clock that describe the gradual slowing down and its relation to the observed phase pattern (Giudicelli et al., 2007; Herrgen et al., 2010; Morelli et al., 2009). The most anterior observable traveling wave of gene expression appears to overlap with the first permanent patterns of segmental gene expression and the emergence of a defined rostrocaudal polarity within the future somite territory (Sawada et al., 2000). This observation is

consistent with the expectation from the Clock and Wavefront mechanism that the segmental pattern is established where the oscillations arrest. In mouse, this transition occurs between S-I and S0 (Morimoto et al., 2005), whereas in zebrafish it appears more posteriorly with respect to the most recently formed somite, between S-II and S-I (Sawada et al., 2000). Surgical rotation of PSM tissue in chick suggests that segments may be ‘determined’ more posteriorly around S-IV (Dubrulle et al., 2001). These segments may appear as determined because oscillations are already too slow at this position in the PSM to allow for a correction of the pattern in the remaining time before the cells become part of a segment.

The arrest of oscillations is the most extreme change possible to the behavior of a single cell in the segmentation clock, and understanding this arrest should reveal key details about the oscillatory mechanism.

FGF, RA and Wnt gradients position the arrest front

In all vertebrates, various members of the FGF and Wnt families are expressed in the tailbud and posterior PSM. A correspondingly graded distribution of FGF protein across the PSM has been observed, as has graded distribution of FGF and Wnt downstream signal transduction components and target gene expression (Aulehla et al., 2008; Dubrulle and Pourquié, 2004). Conversely, RA is synthesized in formed somites and anterior PSM, and correspondingly graded expression of an RA response element transgene has been seen in mouse (Sirbu and Duester, 2006; Vermot et al., 2005). RA signaling appears mutually antagonistic to both FGF and Wnt signaling in the posterior body, and thus the shapes of their opposing gradients are interdependent (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Olivera-Martinez and Storey, 2007; Sirbu and Duester, 2006; Zhao and Duester, 2009).

Disruption of these signaling pathways early in development can lead to axis truncations. Transient perturbations of these pathways have thus yielded the clearest evidence of their roles during

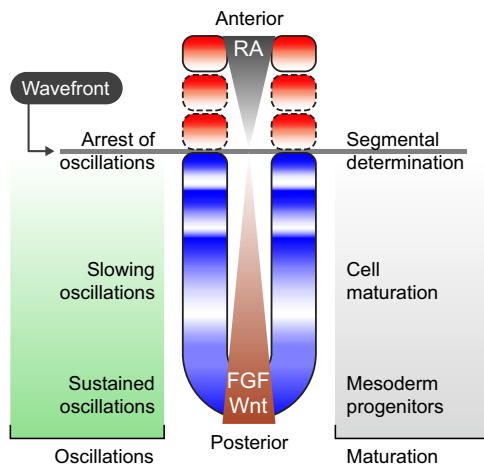


Fig. 6. Global gradients control slowing and arrest of oscillating cells.

An elongating vertebrate axis showing cyclic gene expression patterns in blue/white and arrested segments (dashed boundaries) and somites (solid boundaries) in red/white. Gradients of fibroblast growth factor (FGF) and Wnt (brown) span the tissue from the posterior. An opposing shorter-range retinoic acid (RA) gradient (gray) expands from the recently formed somites. The actual shape of these gradients is not known. Sustained high-frequency oscillations are observed in the posterior region of the tissue. Oscillators gradually slow down as they approach the wavefront of arrest (horizontal line). The panel on the left shows the different stages that single-cell oscillators undergo as they traverse the tissue. The right panel highlights the maturation program of pre-somitic mesoderm cells, running in parallel to segment length specification controlled by the clock.

segmentation. Transient loss of FGF signaling in chick and zebrafish, or Wnt signaling in mouse, or addition of RA in *Xenopus* transiently increases somite length, whereas a transient increase in local FGF signaling in chick and zebrafish or in Wnt signaling in chick decreases somite length locally (Aulehla et al., 2003; Dubrulle et al., 2001; Moreno and Kintner, 2004; Sawada et al., 2001). Changes in somite length are accompanied by alterations in the spatial extent of several pathway target genes in the PSM. These results have been interpreted using the Clock and Wavefront mechanism to indicate that the altered segment lengths are caused by a change to the velocity of the wavefront (Dubrulle et al., 2001; Sawada et al., 2001). However, without quantitative measurements of segment length, axial elongation and somitogenesis period, the possibility remains that alterations to the period of the segmentation clock or changes in embryonic elongation may have contributed to the observed effect.

The current molecular model proposes that a cell in the posterior PSM receives high-level signaling from Wnt and/or FGF that permits oscillations (Aulehla and Pourquié, 2010). As the cell is displaced anteriorly, the levels of signals it receives decrease. At the position in the PSM where the cell crosses below a particular threshold in the signaling gradient(s), the oscillations arrest and the segmental pattern is determined. Consistent with this, overexpression of activated β -catenin throughout the somitic mesoderm lineage, which mimics strongly elevated Wnt signaling, prevents cells arresting in the anterior PSM, leading to a several-fold expansion of the oscillating territory (Aulehla et al., 2008). RA may have the opposite effect, as an increasing concentration correlates with decreasing oscillator frequency. Cells may be able to use opposing gradients of RA and FGF/Wnt to determine their position in the middle of the PSM with higher precision than would

be possible using only the FGF/Wnt gradients from the tailbud.

How is the position of arrest linked to the outgrowth of the tailbud? The shape of a signal gradient in a static tissue is controlled by production of signal at the source, diffusive movement of the signal molecule in the tissue and its degradation (Wartlick et al., 2009). In the elongating embryo, as long as signal-producing cells are maintained in the tailbud, the elongation of the axis should 'drag' the posterior gradients continuously across the PSM. An additional important feature is the flow of cells from posterior to anterior in the PSM, as these cells can transport the signal independently of diffusion. Potentially, this transport mechanism would be sufficient to establish a gradient in a growing tissue. In the mouse and chick, Fgf8 is transcribed only in the tailbud, yet its mature mRNA can be found distributed in a graded fashion more anteriorly in the PSM, suggesting that mRNA decay in cells flowing through the PSM is responsible for the observed graded Fgf8 protein distribution (Dubrulle and Pourquié, 2004). These studies raise a number of questions about the global arrest of oscillations (see Box 6).

From single cells and their synchronization to the global arrest of the oscillations, the segmentation clock ultimately generates a periodic but transient gene expression signal in the anterior PSM. This signal is converted into a fixed and binary rostrocaudal subdivision of the somite, and subsequently into the morphological boundaries of the somite, as described earlier in the review, by a genetic regulatory network (reviewed by Dahmann et al., 2011). Importantly, changes to this network do not appear to affect segment length, somitogenesis period or wavefront velocity, which places this network downstream of the segmentation clock.

Conclusions

The Clock and Wavefront mechanism accounts well for current dynamic observations of somitogenesis ($S=vT$ and $n=d/T$). The three-tier model of the segmentation clock introduced here attempts to describe the interactions of the cellular and molecular processes in the tailbud and PSM that generate the segmental pre-pattern observed in vivo. The concept of the Clock seems to map cleanly onto the single-cell oscillator and local synchronization tiers, and the Wavefront appears to correspond to the global control tier. However, the existence of traveling waves of cyclic gene expression reveals a more complex and subtle relationship. A gradual slowing of the oscillators before arrest implies that the Wavefront is not simply freezing the oscillations of the Clock at a specific position. Rather it must be in continuous communication with the Clock. Thus, the consequence of a gradual arrest is that the Clock and Wavefront cannot be neatly separated as independent processes. Despite progress in our understanding of the regulation of the arrest position by thresholds of gradients of signaling molecules that span the PSM, how these signaling systems act on the pace-making circuits of individual cells to control their oscillation is not known. Understanding how the oscillators are slowed and switched off in greater molecular detail will also provide better insight into the basic oscillatory mechanism.

The causal role of the period of the segmentation clock in determining the anatomy of the vertebrate embryo highlights how little we know about how the period is set. Period appears to be regulated at the single-cell oscillator tier through a pace-making circuit, although we do not understand how. The period is also regulated at the local synchronization tier, and here we have a greater understanding of how this modulation of the single cell

rhythm arises from delays in the coupling. Whether, in addition to their role in slowing and stopping oscillations, the signaling gradients at the global arrest tier affect the period of the segmentation clock in the posterior PSM and tailbud has not been investigated experimentally.

How will progress be made in understanding this challenging system? To obtain an accurate picture of the dynamic behavior of the system at cell and tissue levels, the power of real-time reporters of the segmentation clock must be fully exploited to observe the dynamics of cyclic gene expression in real time. This new information will allow existing models of the segmentation clock to be directly tested, and will spur the development of detailed well-constrained genetic and molecular models. In addition, methods to count the number of molecules in oscillating cells and in spatial gradients will be essential for understanding the sources of noise in the system and, in turn, how precision is achieved despite this noise. The integration of these experimental and theoretical approaches is likely to find application in a wider domain, establishing the segmentation clock as a key model system for understanding dynamic, fluctuating genetic systems in development and disease.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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