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From signalling oscillations to somite formation

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Abstract

Periodic segmentation of vertebrate embryos or somitogenesis is regulated by a dynamic network of signalling pathways. Signalling gradients determine the spacing of the forming segments, while signalling oscillations, collectively termed the segmentation clock, ensure their regular timing. Since the segmentation clock is a paradigm of signalling dynamics at tissue level, its mechanism and function have been the topic of many studies. Recently, researchers have been able to analyse and quantify these signalling dynamics with unprecedented precision, revealing the complexity of interlinked oscillations and tissue-wide dynamics throughout development. Initial studies have shown how the interplay between signalling dynamics and cellular mechanics drive the periodic formation of segments. Looking ahead, new techniques such as *in vitro* stem cell-based models of (human) embryonic development will enable detailed investigations into the mechanisms of somitogenesis.

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Introduction

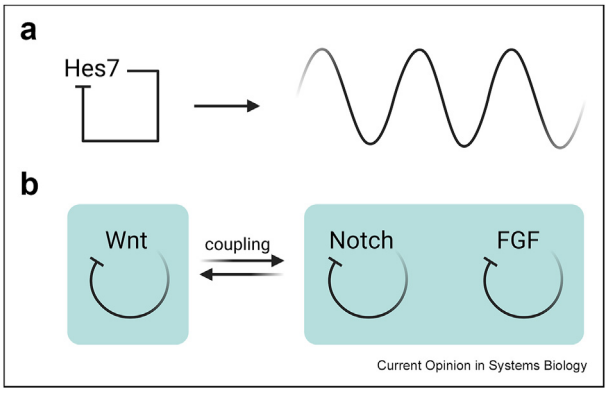
During vertebrate development, a dynamic network of signalling pathways ensures the periodic segmentation of the continuously growing presomitic mesoderm (PSM) into blocks of tissue. These somites are positioned on the left and right side of the forming neural tube and give rise to vertebrae, axial muscles, brown fat, and skin [1]. A combination of signalling oscillations and gradients is thought to determine the timing and spacing of segment formation. Regressing FGF and Wnt gradients emanating from the posterior tip of the embryo towards the PSM, and an antagonistic retinoic acid gradient regulate the space where new segments can form. Signalling oscillations periodically allow segment formation in the competent region set by the gradients [2–5]. This is consistent with the 'clock and wavefront' model proposed nearly 50 years ago [6]. According to this model, the combined activity of regressing wavefront, i.e. gradients, and the clock defines the size of forming segments. Apart from this, variations of this model and other models have been suggested to explain somitogenesis, such as the phase-shift, reaction-diffusion and opposing gradients models (reviewed in depth in Ref. [7]). Even though decades of research have revealed several components of the segmentation clock and genes relevant for segmentation (reviewed in further detail in Refs. [8,9]), many questions on the mechanistic details remain unanswered. With the recent development of new technologies for the analysis and manipulation of signalling in tissues, we are now beginning to dissect somitogenesis in depth. Here, we will discuss the latest findings on the composition of the segmentation clock, the complexity of wave dynamics travelling through the tissue and the process of somite formation with a main focus on mammalian somitogenesis.

The segmentation clock as dynamic network of interacting oscillators

The first evidence for the existence of a segmentation clock is based on the analysis of homologues of fly segmentation genes in vertebrate embryos, which showed periodic expression of these *cyclic genes* in tissue undergoing somitogenesis [10]. Since such genes are downstream targets of signalling pathways, this hinted towards the presence of signalling oscillations in segmenting tissue. A delayed negative feedback mechanism has been suggested to drive these oscillations (Figure 1a). Even though the components of the molecular clock have not been fully defined yet, the family of basic helix loop helix (bHLH) transcription factors is critical [11,12]. In mouse, expression of the bHLH protein Hes7 is induced by Notch signalling and acts as repressor of its own expression and that of other genes (Figure 1a) [11,13].

While the presence of a segmentation clock seems to be conserved across vertebrate species, the details of the identity of oscillatory pathways and genes known to date varies [14]. In mice the segmentation clock consists of

Figure 1



Coupled oscillating signalling pathways make up the mouse segmentation clock. (**a**) Oscillations are created by a delayed negative feedback loop. A critical clock component is the transcription factor Hes7, whose expression is induced by Notch signalling. Hes7 itself functions as transcriptional repressor of its own expression, which leads to periodic inhibition of Hes7 expression and thus oscillations. (**b**) In mouse embryos, the segmentation clock consists of Wnt, Notch and FGF signalling. While Notch and FGF signalling are dependent on the Hes7 feedback loop, Wnt signalling oscillations are thought to be independent of this. However, oscillations of the three signalling pathways still interact with each other.

cyclic Wnt, Notch, and FGF signalling pathways [13,15–18]. In zebrafish, Notch signalling has long been found to be oscillatory [19]. Additionally, a recent study has revealed oscillations in levels of phosphorylated ERK downstream of FGF signalling. In clock-deficient zebrafish mutants, pulsatile FGF or MEK inhibition was able to rescue the segmentation clock and lead to well-defined epithelialized somites [20]. How periodic inhibition of ERK signalling affects oscillations of other segmentation clock components has to be studied in the future. Additionally, Tbx6, a Wnt target gene, has been indicated to oscillate in zebrafish [21]. Interestingly, target genes of those pathways have also been found to undergo oscillatory expression in model systems of human segmentation [22–25]. In human, the general mechanism of oscillations and gradients seems to be conserved and further research will reveal the mechanistic details of human somitogenesis. When expressing dynamic Hes7 signalling reporters in those human model systems, the period of the human segmentation clock could be quantified to be approximately 5 h, a similar period as had been predicted previously for sequential somite formation based on human embryos [22–25]. The difference in the period between mouse (2.5 h) and human (5 h) somitogenesis has been attributed to approximately 2-fold slower biochemical reactions in human cells [26], a distinguishing feature that has also been observed in *in vitro* differentiation of human or mouse pluripotent cells towards the neural tube [27]. Differing metabolic rates between the two species might account for the differences in developmental timing [28]. A study comparing six different species found that the transcriptional profile scales with developmental timing, which is conserved across cell types [29]. Such studies can give insights into species-overarching mechanisms that drive oscillatory dynamics in development.

Much research to date has focused on Notch signalling oscillations, which are found in all vertebrate species investigated thus far. However, transcriptome analyses of mouse embryos at different phases of the oscillation cycle, and of synchronized *in vitro* cultures of human segmentation, paint a more complex image of the segmentation clock [16,23,30]. Central among transcriptionally identified genes are oscillations in Notch, Wnt and FGF signalling pathways, whose role in somitogenesis has indeed been confirmed in mouse embryos [15,31]. Besides Notch, Wnt, and FGF, downstream target genes of various other signalling pathways and cellular components showed periodic expression, among these TGF-β and Hippo signalling [16,23,32]. While the focus in somitogenesis research on Notch, Wnt, and FGF signalling is likely historically grown, with crosstalk between signalling pathways in various model systems and cell types, it is not surprising that oscillations in one molecular pathway affect oscillations in a multitude of pathways. Indeed, when modulating Notch signalling oscillations of the mouse segmentation clock using microfluidics, Wnt signalling oscillations follow – and *vice versa*, indicating their reciprocal coupling (Figure 1b) [31,33]. Hes7 is suggested to be integral to this coupling between the pathways. Notch signalling induces Hes7 expression, which is expressed in an oscillatory manner, which then appears to drive oscillations in Notch and FGF signalling [18]. Direct modulation of individual pathways or individual proteins should reveal the relevance of each of these oscillators within the network of the molecular clock and in the segmentation process in the future.

Besides oscillations of the segmentation clock, there are several other periodic events in an organism. Whether and how these different types of oscillators are linked with the segmentation clock in the developing embryo is not entirely clear. An overarching circadian rhythm with a period of 24 h has been suggested to be forwarded from the mother to the embryo during development [34]. Indeed, expression of the circadian clock components CLOCK/BMAL1 has recently been found to directly affect segmentation clock dynamics through upregulation of *hes7* [35]. Moreover, Notch signalling oscillations are correlated with the oscillations of the cell cycle [36,37], even though the segmentation clock proceeds under cell cycle inhibition [38]. Future research should address whether cell proliferation is merely noise to the segmentation clock [36] or is an active component of somitogenesis [39,40].

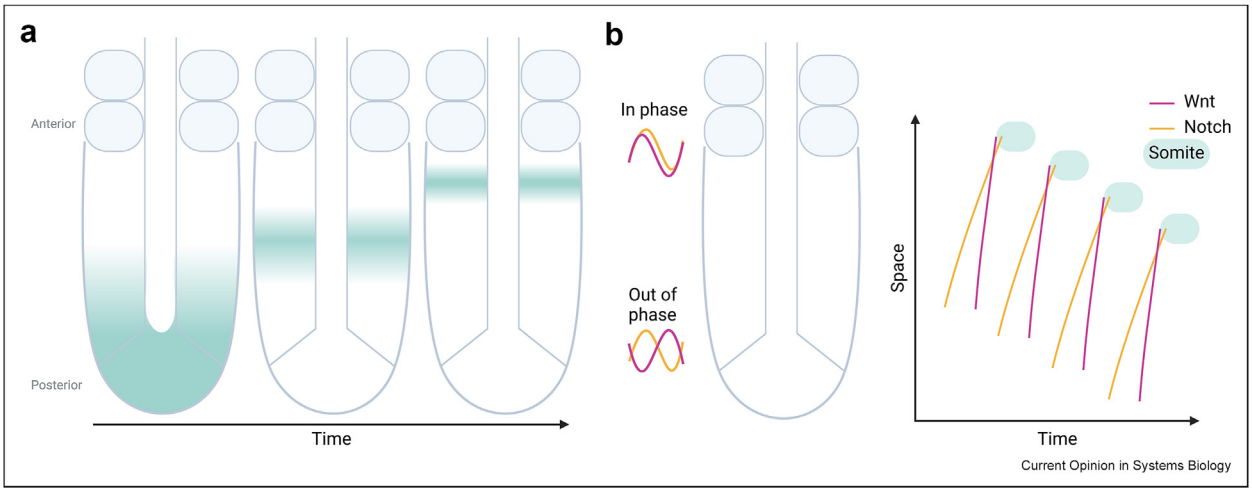
Tissue-wide coordination of the segmentation clock

At the tissue level, neighbouring cells are coupled and the oscillations between them are phase-shifted along the anterior-posterior axis, which leads to kinematic waves travelling through the tissue from the posterior to the anterior end of the PSM (Figure 2a). Depending on species, wave dynamics can change over space and time: In zebrafish, segmentation from anterior to posterior proceeds with a different velocity than the timing of new signalling waves emerging in the posterior. Over time this leads to a decrease in the number of waves travelling through the tissue at a given time [41]. In contrast, in mice the phase gradient is maintained more stably at approximately 2π over developmental time, i.e. a new wave starts in the posterior roughly when the previous wave has reached the anterior PSM [2,42,43]. Over space, the speed of the Notch signalling wave decreases along the tissue and the phase-relationship between Notch and Wnt signalling oscillations changes from out-of-phase in posterior to in-phase in anterior PSM (Figure 2b) [14,31]. When experimentally changing the phase-relationship to out-of-phase in anterior PSM, segmentation and differentiation are impaired, which highlights the importance of the relative dynamics between the two oscillating pathways for somitogenesis [31]. The importance of relative timing between different oscillators in other species including zebrafish still has to be addressed [7,21] Other more general processes might also potentially modulate dynamics, such as post-translational modifications, alternative splicing or even tissue mechanics [44–46]. For example, stability of Lunatic Fringe (Lfng) mRNA downstream of Notch signalling can be regulated by miRNAs [47] (Reviewed extensively in Ref. [46]).

Questions regarding the relevance of such complex tissue-wide dynamics in the different species, how the dynamics are regulated and how signalling gradients modulate them remain to be investigated. For instance, it has recently been suggested that – at least in human "gastruloids" – diffusible signals such as Wnt and FGF play a role in propagation of the oscillations of the segmentation clock [25]. In a recent preprint, single-cell oscillations in segmenting zebrafish embryos have been quantified both *in vivo* and in ex vivo cultures of single cells. Under both conditions, changes in oscillation dynamics and induction of differentiation progressed similarly. This indicated that an intrinsic "molecular timer" might control this progression, which might then be fine-tuned by extrinsic factors, as cells move along the PSM [48]. Future studies should reveal the identity of this timer and address whether such a timer functions similarly in other species.

While the signalling waves traversing the PSM during ongoing segmentation have been well described using real-time imaging of dynamic signalling reporters, the start and end of somitogenesis are less well understood. In chick, waves of Hairy and Lfng gene expression have been seen in the primitive streak before the onset of segmentation [49]. Recently, the first sign of the segmentation clock and signalling waves has also been seen in E6.5 mouse embryos, when Notch downstream target *Lfng* appears as a pulse in the primitive streak [43]. Distinct oscillations arise at late bud stage (approximately E7.5), together with a gradual establishment of spatiotemporal wave patterns. While the initial *Lfng* pulse was observed even under Notch inhibition (consistent with previous findings in chick [49] and fish [50]) or *Hes7* knockout, the formation of waves required

Figure 2



Complex signalling wave dynamics ensure periodic somite formation. (**a**) Signalling waves emanate from the posterior tailbud of vertebrate embryos and travel towards the anterior side of the PSM, where segmentation occurs. (**b**) In mouse embryos, Wnt and Notch signalling oscillate out-of-phase in the posterior and in-phase in the anterior PSM. The phase relationship between Wnt and Notch signalling is important for proper segmentation.

*Hes*7 activity [43]. At the end of somitogenesis, termination of the segmentation clock has been linked to expression of posterior Homeobox (Hox) genes (9–13) in chick embryos [51]. Hox genes determine the axial identity of the forming somites and get expressed in a colinear fashion from anterior towards the posterior [52]. Progressive expression of posterior Hox genes in chick embryos is thought to gradually downregulate *Brachyury* expression via repression of Wnt/β-catenin signalling, and reduce PSM cell motility through downregulation of FGF, together slowing down axis elongation and ultimately terminating segmentation [51]. Before the termination of somitogenesis in chick, Notch signalling dynamics were found to slow down and become restricted to the rostral PSM before completely disappearing, however the mechanistic link between Notch signalling dynamics and Hox genes remains unknown [53]. In zebrafish, Hox13 has been shown to drive Wnt and *Tbxt* expression for maintenance of progenitor cells in the tailbud and axis elongation [54–57]. These posterior Hox genes are proposed to create a niche for the neuromesodermal progenitor (NMP) pool, but also deliver positional information upon transition towards the PSM [32,54]. T-box transcription factors T and Eomes downstream of Wnt and Hox genes were also indicated to play distinct roles in providing positional information and driving the specification of presomitic mesoderm [58,59] The exact function of posterior Hox genes and their downstream targets in somitogenesis and the effect on the segmentation clock in different species still has to be clarified.

Somite formation

Somite formation occurs in a highly regulated, species-specific manner that is not yet fully understood. On the one hand, somitogenesis encompasses a periodic mesenchymal-to-epithelial transition (MET) to form epithelialized blocks of cells with a mesenchymal lumen, surrounded by a layer of fibronectin-rich matrix. On the other hand, concomitantly with segmentation, PSM cells differentiate into somitic cells, followed by diversification towards rostral or caudal identity and subsequent differentiation into sclerotome and dermomyotome (Figure 3) [60]. The basic understanding today is that low levels of Wnt and FGF signalling gradients are necessary to allow somite formation. Overactivation of either shifts the newly-forming segment boundary anteriorly [3,15,42], whereas periodic Notch activation is thought to induce segmentation [18]. How pre-patterning by signalling gradients and oscillations affects somitogenesis and how MET is regulated at subcellular level is not entirely clear.

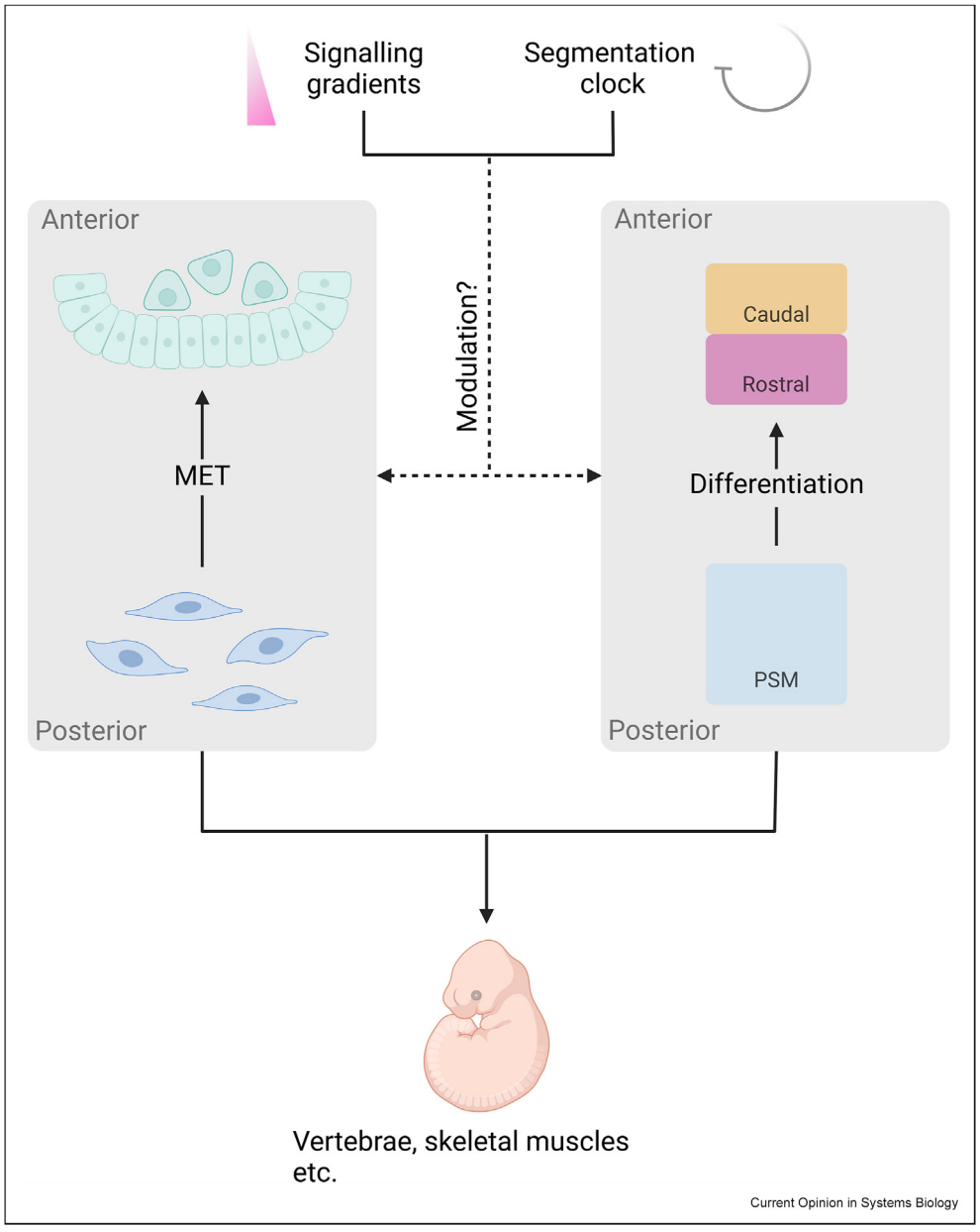
Similarly to other examples of MET, there is thought to be a change from N-Cadherin to E-Cadherin expression and matrix adhesion along the tissue, though the exact mechanism is not yet understood [61–63]. It has been hypothesized that epithelialization and cell polarization in somitogenesis are gradual events, which would be supported by the fact that various ECM components show a graded expression pattern in chick [64]. In stem cell-based *in vitro* models of somitogenesis, the addition of extracellular matrix components such as fibronectin is necessary to induce somite formation, presumably via fibronectin-mediated integrin signalling [30,65–67]. Surprisingly, a recent study shows that knocking out N-cadherin in gastruloids allows them to bypass this necessity, and segment by themselves [68]. Over the years, some factors promoting MET in somitogenesis have been identified. A central component is the transcription factor *Mesp2*, which is periodically expressed in a Notch-dependent manner in anterior PSM, where FGF signalling is low [18,60]. *Mesp2* has been implicated in both somite formation itself by inducing alternating Eph-Ephrin signalling at the segment boundary and the rostrocaudal patterning of somites [13,60,69,70].

Despite these intricate interactions between MET and patterning, segmentation (i.e., epithelization) and differentiation induced by signalling oscillations can be experimentally decoupled [20]. For instance, when changing the Wnt-Notch phase-relationship to out-of-phase oscillations in anterior PSM (see above), segmentation is impaired, whereas *Mesp2* expression is still induced [31]. Furthermore, the signalling dynamics driving somitogenesis and induction of *Mesp2* expression can proceed in gastruloids – embryo-like structures recapitulating somitogenesis – without apparent segmentation [30]. Only when placed into a low percentage of extracellular matrix components, somite formation is induced [30,67]. These studies highlight that the activity of *Mesp2* is necessary, but not sufficient for somite formation. Conversely, even in the absence of a properly functioning clock and thus without initial boundary formation, animals develop with a clearly distinguishable – albeit mis-shaped – ribcage [71–73]. This suggests that segmentation is not a prerequisite for downstream differentiation into sclerotome and dermomyotome. It has therefore been proposed that the clock coordinates the exact spacing and timing of the segmentation process, rather than enabling it [72]. In zebrafish, it has recently been shown that after initial somite formation segments are not yet accurate in either shape or size, and that this is subsequently adjusted by tissue mechanics, in particular somite surface tension, to establish left-right symmetry [74,75]. Thus, while signalling dynamics play an essential role in somite formation, their exact contribution and the interdependence with tissue mechanics have yet to be resolved.

Discussion

Over the last years, vertebrate somitogenesis has been investigated in great detail and several components have been identified to play a role in the sequential formation

Figure 3



Somite formation requires mesenchymal-to-epithelial transition and differentiation. For proper somite formation two processes have to occur: (1) Mesenchymal-to-epithelial transition (MET) of presomitic cells at defined locations in the PSM leads to the formation of an epithelialized somite boundary and a mesenchymal lumen. (2) Differentiation of PSM cells into somite cells with a rostral or caudal identity ensures the subsequent differentiation of somites. Both mechanisms are crucial for the following development of structures such as vertebrae, skeletal muscle and dermis. Signalling gradients and the segmentation clock modulate both MET and differentiation.

of somites. Such studies also highlight the complexity of somite formation and raise several questions on the mechanism of somitogenesis, such as: (1) What is the functional relationship and hierarchy between the oscillating signalling pathways, gradients and mechanics at single-cell and tissue level as well as their downstream effect? (2) What controls the changing wave dynamics along the tissue and how do signalling gradients affect these dynamics? (3) Which cells maintain stable oscillations in the tailbud, while others slow down and why? (4) When, where, and how does the segmentation clock start over developmental time and how does it terminate? (5) What is the link to other cellular oscillators such as the circadian clock or the cell cycle?

Much of our knowledge about the mechanism of somitogenesis to-date is based on the analysis of fly gene homologues, knockout mice or selected candidates. While the functional significance of these genes and signalling pathways has indeed been confirmed, the question remains whether all key components and relevant downstream targets have been identified yet. Using more unbiased approaches to analyse the transcriptome, a large group of cyclic genes has indeed been identified in mouse embryos nearly 20 years ago. Three broad clusters of cyclic gene expression patterns in Notch, FGF, and Wnt signalling were defined at the time [16]. However, more than 3000 genes were found to be cyclic in their initial analysis. Similarly, in the human segmentation clock a huge number of genes appears to be expressed in an oscillatory manner [22,23]. Re-analysing these datasets in combination with available single-cell RNAseq will provide a much more detailed view of the processes in cells of the PSM, to further elucidate the network of genes that underlie the segmentation clock.

It has already become apparent that there are differences in the mechanistic details between species, and knowledge from one species should not be transferred directly to another without confirmation. An in-depth comparison between different vertebrate species and with other segmenting animals might provide answers on the evolution of the segmentation process in general and the segmentation clock in particular a.

Excitingly, advanced technologies have recently been established and continue to be developed that will allow an unprecedented investigation of the segmentation clock in the future. Highly sensitive omics technologies allow for the identification of expressed genes and downstream targets as candidates for further analysis [76–82]. Light-sheet imaging in combination with machine learning enables the quantification of signalling oscillations and cellular processes at single-cell resolution [43,48,83]. To dissect the function of oscillating signalling pathways or individual genes, technologies such as optogenetics and microfluidics can be applied to modulate signalling oscillations with high spatiotemporal precision [31,33,84,85]. Finally, stem cell-based embryo-like structures serve as *in vitro* models of somitogenesis and can be generated in larger numbers to enable the implementation of screens and biochemical investigation of the segmentation clock [25,86–88]. Such embryo-like structures even allow scientists to study the human segmentation clock at molecular detail [23,24,30,67,89–91]. With an increasing amount of quantitative data describing the segmentation process, theoretical modelling will be essential to integrate all findings into a common framework. Applying these technologies and combinations thereof makes an interdisciplinary approach and a closer interaction with scientists of different disciplines essential.

Thus, while our understanding of the segmentation clock has advanced considerably over the last years, many open questions remain regarding its mechanism. With new technologies to quantify, perturb, and analyse the signalling dynamics of the segmentation clock at mechanistic detail, exciting times certainly lie ahead of us.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- • of special interest
- •• of outstanding interest

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