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The mechanism of somite formation in mice

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Somitogenesis is a series of dynamic morphogenetic events that involve cyclical signaling. The periodicity of somitogenesis is controlled by segmentation clock operating in the presomitic mesoderm (PSM), the precursor of somites. Notch signaling plays important roles not only in the segmentation clock mechanism but also as an output signal of the clock to induce *Mesp2* transcription that controls somite formation. In the present review, recent advances in the understanding of the molecular mechanisms underlying the translation of clock information into the spatial patterning of segmental somites in mice are discussed. Particular attention is paid to the interplay between two the distinct signaling pathways of Notch and FGF and the Mesp2 transcription factor acting as an effector molecule during mouse somitogenesis.

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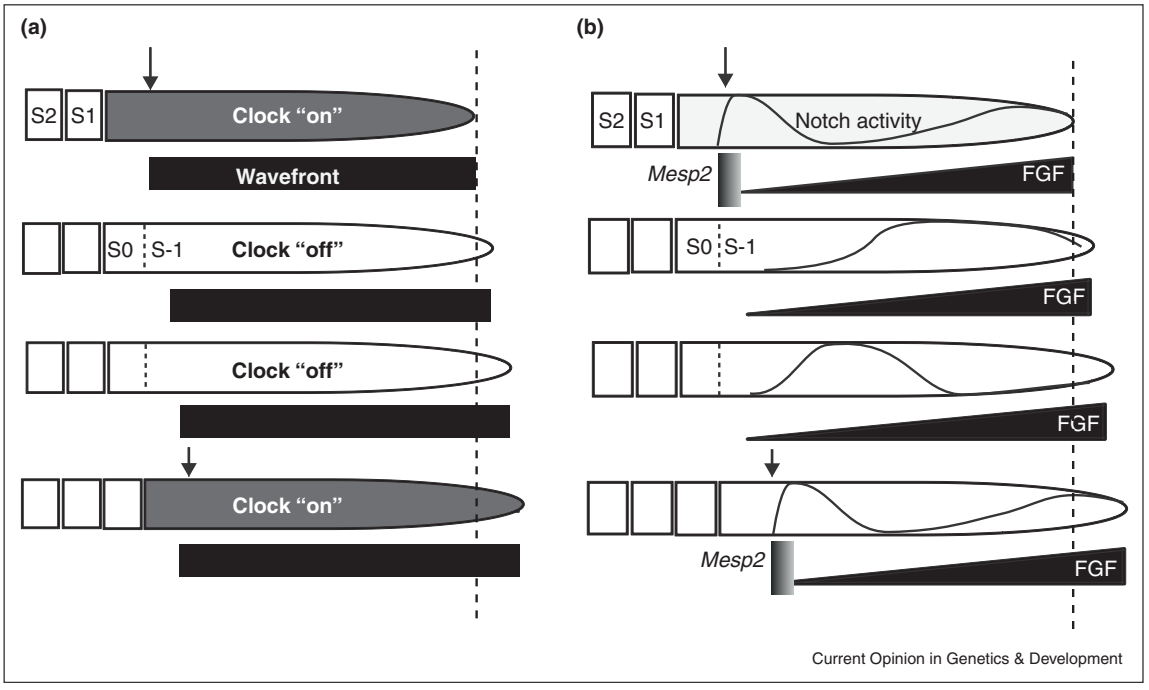
Introduction: clock and wavefront model

A fascinating feature of somitogenesis is its spatio-temporal regulation [1]. Somite formation in the mouse commences from embryonic day (E) 8.0 and ends at E13.0 during embryogenesis. The primitive streak during the gastrulation stage and the tailbud at later developmental stages are the cell sources that produce the presomitic mesoderm (PSM). The PSM is then sequentially subdivided into blocks of epithelial cells to form somites. This occurs from the anterior end of the PSM on both sides of the neural tube. The mechanism underlying the periodic formation of the somites has been subject of considerable interest in the field of theoretical biology and led to the proposed 'clock and wavefront model' some time ago when no molecular information was yet available [2]. This theoretical model encompasses two components: a clock that measures and determines the time for somite formation, and a wavefront that provides spatial positional information and defines the point where the segmentation program initiates [3]. The wavefront is defined as the region at a constant distance from the posterior end of the tailbud. The wavefront regresses in the posterior direction along the extension of the body axis. Hence a segmentation point is defined only when the wavefront encounters cells in which the clock is in an 'on' state (Figure 1a).

As predicted by the clock and wavefront model, it has been shown that each PSM cell has a clock, the molecular nature of which is now quite well understood in the mouse as a negative feedback mechanism centered on the activities of the Hes7 transcription factor [4]. *Hes7* is initially activated at the posterior end of the mouse embryo by FGF signaling and comes under the control of Notch activity, in turn suppressing its own transcription to generate an oscillatory Hes7 expression pattern [5•]. Notch activity also activates another Notch target *Lunatic fringe*, encoding a modulator of the Notch receptor, which suppresses Notch activity [6,7]. Hence, Notch activity oscillates in the PSM and serves as a so-called Notch clock oscillator [8•]. The wavefront is often referred to as a maturation wavefront, since the PSM cells are maintained as an immature mesenchymal state in the posterior PSM and acquire the competence to form an epithelial somite once they pass through the wavefront. The molecular identity of the wavefront has long been a controversial issue. Initially, Fgf8 was proposed to encode wavefront activity because the experimental manipulation of its levels in cultured chick and zebrafish embryos caused corresponding shifts in the position of the determination front [9,10]. However, mouse embryos lacking Fgf8 in the PSM still undergo somitogenesis [11]. Mice homozygous for null mutations in other FGF ligand genes also show no early somitogenesis defects (Fgf3, Fgf5, Fgf15, Fgf17, and Fgf18), or die before somitogenesis initiates (Fgf4) [12]. Wnt has also been proposed to contribute to wavefront activity, since the manipulation of canonical Wnt signaling also causes corresponding shifts in the determination front [13,14]. However, FGF signaling is also affected in Wnt loss-of-function embryos.

To better understand the actual role of FGF signaling in somitogenesis, a conditional strategy has been employed to inactivate both Fgf4 and Fgf8 in the PSM. This resulted in dramatic shifts in the determination front and the premature differentiation of the PSM [15••]. Moreover, the restoration of Wnt signaling to these mutants did not restore the determination front, demonstrating that these two FGF molecules constitute the

Figure 1



Models based on the 'clock and wavefront model' concept. **(a)** Model based on the classical clock and wavefront hypothesis. Segmentation occurs when the clock is turned on in the wavefront that then regresses at a constant rate in tandem with the posterior extension of the tailbud. **(b)** After the identification of molecular components of the clock and wavefront, the model was modified by incorporating the corresponding molecular mechanisms. The active Notch oscillation wave acts as the clock, which defines the timing of segmentation initiation. The clock turns on the expression of Mesp2 which is required for somite segmentation, when FGF signals reach a subthreshold level.

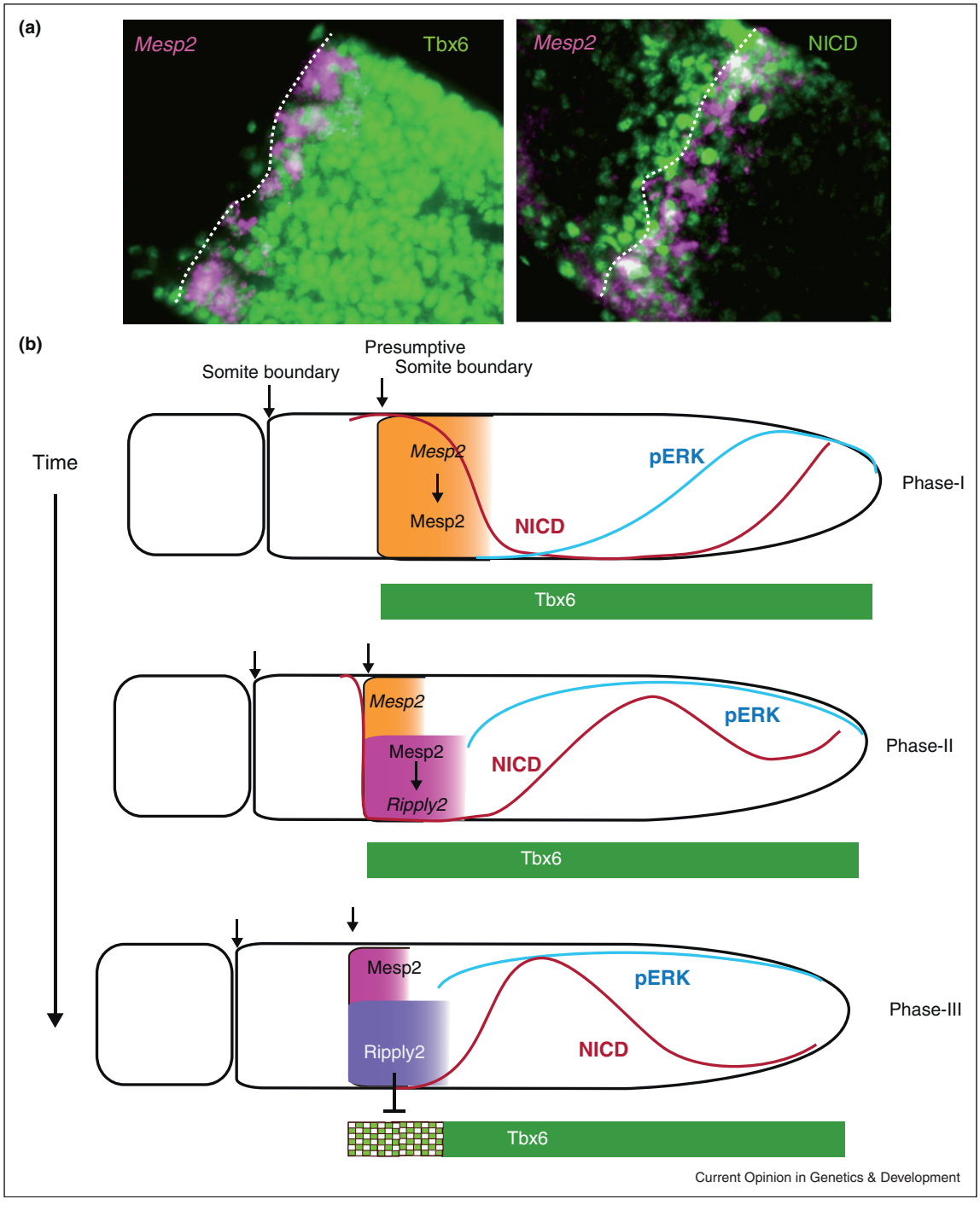
proposed wavefront activity that maintains the PSM in an undifferentiated state. In a new model based on molecular identification, the pace of the segmentation is proposed to be dependent on the clock via the control by cyclic genes such as Notch signaling molecules, whereas the location of segmentation is defined by the wavefront established by Fgf8 (Figure 1b). However, this hypothesis faces challenges due to a new finding that the FGF signaling also oscillates in the PSM as it will be discussed in the next section. The link between clock and wavefront has been provided by analyses of the Mesp2 transcription factor that signals the initiation of the segmentation program [16]. In the current review, recent advances in the understanding of the critical events underlying the wavefront, segmental border formation and rostral-caudal pattering within a somite are presented and discussed.

The mechanism of periodical *Mesp2* induction to establish a segmentation point

The most reliable molecular marker of segmentation initiation is the transcriptional activation of *Mesp2*. *Mesp2* expression is periodically observed in the S-1 region in conjunction with the alteration of the domain from a one somite length to a one-half somite length, and then disappears before the next round of expression [17]. Initially, the Tbx6 transcription factor was revealed to directly bind to the Mesp2 enhancer [18•] and the requirement for Tbx6 binding was confirmed by the analysis of an enhancer-specific knockout mouse, in which *Mesp2* expression is diminished and a segmentation defect similar to that of the *Mesp2*-null mouse is observed [19]. High resolution in situ staining analyses have clearly shown that the anterior border of the *Mesp2* expression domain accords with the anterior border of Tbx6 [20••]. However, the Tbx6 expression domain is extended to the entire PSM and co-expression with *Mesp2* is only observed in the anterior limit (Figure 2a, left). Hence, other factors are required to drive the periodic expression of *Mesp2* and define the posterior border of the *Mesp2* domain.

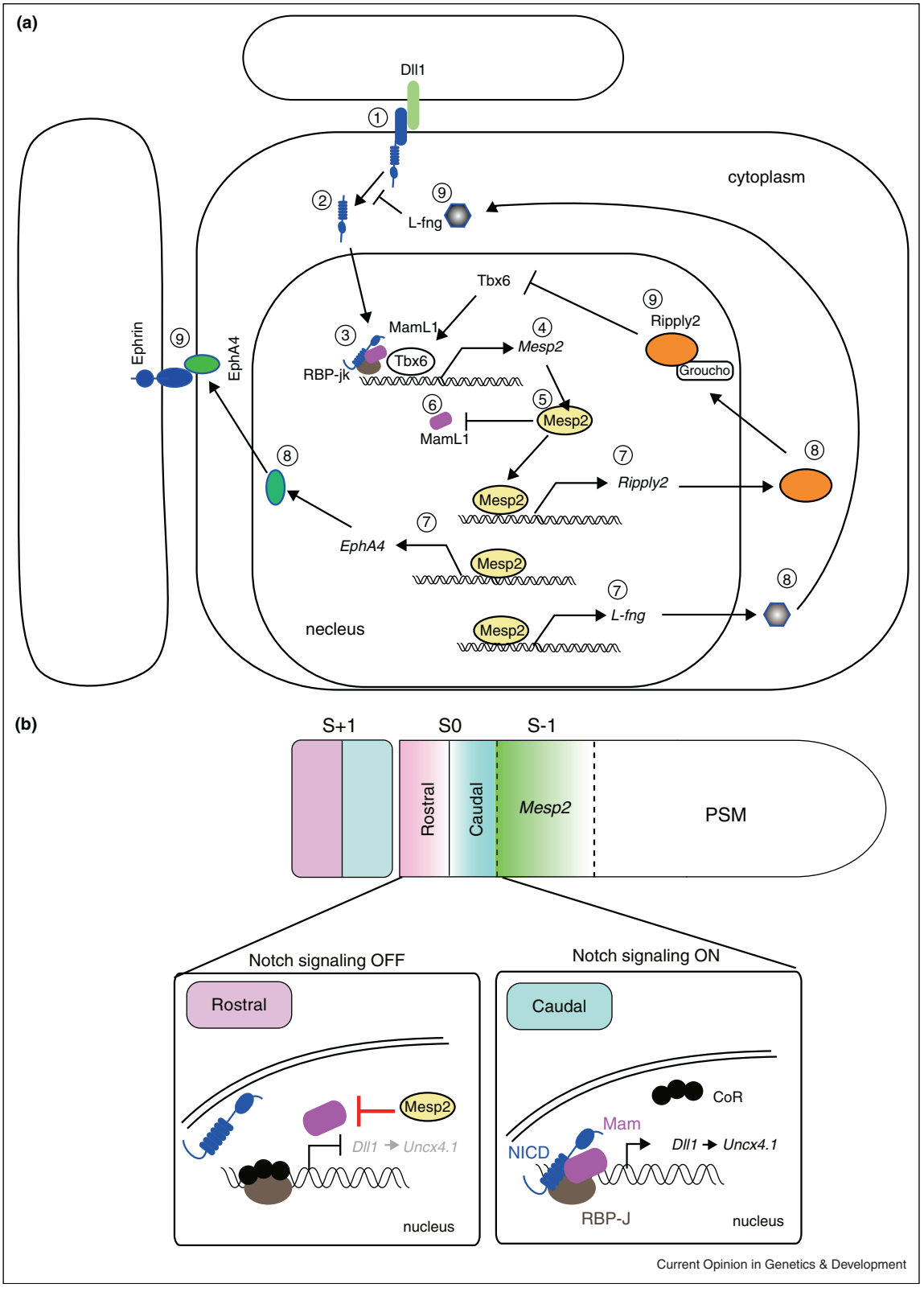
The results of reporter assays have indicated that Notch signaling positively regulates the *Mesp2* enhancer in a Tbx6-dependent manner in cultured cells. This finding is supported by the fact that *Mesp2* expression is strongly suppressed in Notch mutant embryos such as Dll1-null and RBP-jk-null. In addition, in situ expression analyses have revealed that Mesp2 transcription occurs only in cells showing Notch activity in the Tbx6-expressing domain (Figure 2a, right), indicating that the periodic activation of Mesp2 is brought by oscillating Notch

Figure 2



Key events in segmental border formation. **(a)** Mesp2 transcription occurs at the anterior end of the Tbx6 domain (left) and also when Notch signals are active (right). Double staining for *Mesp2* transcripts (magenta) by in situ hybridization and immunological detection of Tbx6 protein (green, left panel) or active Notch (green, right panel) are shown. Dotted lines indicate the presumptive next segmental borders. **(b)** Schematic depiction of the sequential events involved in segmental border positioning based on the new clock and wavefront model. In phase-I, when the Notch active domain reaches the anterior part of the Tbx6-positive PSM and is released from the pERK active domain, *Mesp2* transcription is turned on. In phase-II, the *Mesp2* expression domain becomes restricted to the presumptive rostral part of S-1, whilst Mesp2 protein begins to be accumulated in entire S-1. This then turns on the downstream target gene, *Ripply2*. In phase-III, when *Mesp2* transcripts are absent, Tbx6 protein expression is strongly suppressed in the Mesp2-expressing domain via the function of Ripply2. *Mesp2* transcription is repressed in the posterior PSM when pERK is positive even if Notch activity is elevated.

Figure 3



Genetic networks operating in the cell in the rostral compartment of S-1. **(a)** Once Notch1 receptor is activated (①), the intracellular domain (NICD) is cleaved (②) and enters the nucleus. NICD then forms a complex with MamL1 and binds the *Mesp2* enhancer in conjunction with Tbx6 protein (③). Upon activation of Mesp2 (④), MamL1 is destabilized (⑤), which leads to the suppression of Notch. Mesp2 also activates several target genes (⑥)

activity working as a clock output. The most pertinent question that arises from this is how the posterior border of the *Mesp2* expression domain is defined. In the absence of FGF signaling, this domain shifts into the posterior PSM, indicating that *Mesp2* expression is suppressed in this region via FGF. Recently, the relationship between Notch and FGF signaling was further addressed via detailed expression analyses together with in vivo imaging technology [21••]. Intriguingly, it was demonstrated that the activity of the FGF signal effector, pERK, also oscillates in the posterior PSM with different dynamics from that of Notch signaling; NICD oscillation displayed a band propagation pattern from the posterior to S0, while pERK oscillation displayed an on–off pattern in the posterior to S-2 (Figure 2b). It was further found that Mesp2 activation via Notch activity becomes possible only when pERK signaling is low during the oscillatory cycle. The fact that pERK signaling also oscillates in the PSM somewhat alters some of the previous concepts regarding the wavefront that were explained as an FGF gradient. The wavefront should not be defined by the stable activity of FGF signal itself, but rather it might be considered as a condition to provide for proper Notch signal propagation.

Therefore, the posterior limit of the Mesp2 activation domain is likely to be defined by the Notch active domain, which thus represents a new clock and wavefront model (Figure 1c), in which Notch oscillators define the prospective somite region (space), whilst Fgf oscillators regulate the pace of segmentation (time). These two oscillators may be linked via the function of Hes7 that works as a positive regulator for both Notch (through the suppression of lunatic fringe) and FGF (by blocking Dusp4) signaling pathways [5•].

It is noteworthy that Mesp2 is not only a marker but also plays essential roles in somite segmentation. In the absence of Mesp2, the protein expression of Tbx6, but not its mRNA expression domain, expands anteriorly, indicating that Mesp2 is required for the suppression of Tbx6 protein in the Mesp2-expressing domain (S-1) [20••]. This in turn generates the next Tbx6 anterior border which will be the next segmental border. At least in mice, the Tbx6 suppression process is mediated by an ubiquitin-protease pathway and by Ripply1/2 downstream targets of Mesp2 since Ripply1/2 knockout resulted in the anterior expansion of Tbx6 in a manner similar to that found in the Mesp2-null embryo [22•]. Therefore, Mesp-Ripply makes a negative feed-back loop, thereby Mesp2 expression is indirectly suppressed by the Ripply function (Figure 2b). This cascade is known to be influenced by Retinoic Acid through RARE (retinoic acid responsive element) [23]. However, the response in Mesp paralogs in different species varies based on the presence or absence of RARE. It is likely that RA has multiple input points in the feedback loop between Mesp and Ripply genes, with certain species favoring RA input on the Ripply side (zebrafish) while others favor input on the Mesp side (frog and perhaps fugu) although no clear information regarding mouse counterparts is reported so far.

The segmental border is sequentially positioned through the cooperative function of Tbx6 and Mesp2. However, the mechanism by which the morphological segregation of the somite is achieved following the border definition remains unresolved. One of the Mesp2 target genes, *EphA4*, which encodes a transmembrane protein kinase, is implicated in this process [24] via its interaction with ephrin proteins expressed in juxtaposed cells (Figure 3a). Although loss of function experiments in the mouse have not yet provided direct evidence for any positive involvement of specific Eph or ephrin molecules in the segregation process, the ephrin reverse signaling pathway has been implicated in chick somitogenesis [25].

The mechanism of somite patterning (rostro-caudal polarity)

Another intriguing feature of the segmented somite is its clear compartmentalization into rostral and caudal parts, which is accompanied by differential gene expression patterns and lineage restriction upon differentiation [26]. This intra-somitic patterning is also established by a mechanism mediated by Mesp2, since Mesp2-null embryos develop completely caudalized somite derivatives [17]. In addition, Notch signaling activity is a required determinant of the caudal identity of the somite since its absence in the caudal compartment results in a rostralized phenotype [27] and the ectopic expression of active Notch leads to the complete caudalization [28]. Hence, the relationship between Mesp2 and Notch signaling had remained a critical issue to be addressed. On the basis of the fact that Mesp2 expression is ultimately restricted to the rostral compartment of a somite, it appears that Mesp2 suppresses Notch activity in the rostral compartment [17]. Initially it was thought that L-fng induced by Mesp2 is involved in the suppression of Notch activity in the rostral compartment [8•]. However, the functional significance of L-fng in this regard has been discounted, since normal somitogenesis occurs even in the absence of L-fng under the control of Mesp2 [29].

which are involved in somitogenesis (⑦). Ripply 1/2 proteins are involved in the suppression of Tbx6 expression. EphA4 may interact with ephrin and induce Eph reverse signaling, leading to morphological segregation. L-fng is also an expected Mesp2 target involved in the suppression of Notch signaling. **(b)** The rostral-caudal compartment is established by the differential Notch activity caused by MamL1 stability. Once *Mesp2* is transcribed and the protein product localizes in the rostral compartment, the destabilization of MamL1 occurs, which results in the destruction of NICD-active complex. In the caudal compartment, Notch activity is maintained by a positive-feedback of Notch signaling and caudal genes such as *Dll1* and *Uncx4* are transcribed.

The Notch activity wave does not stop properly without Mesp2, as a result of which extended Notch activation is observed in the anterior direction causing a transformation of the rostral compartment into its caudal counterpart. However, this is primarily due to the up-regulation of the Notch ligand Dll1 caused by increased Tbx6 expression in the anterior part [30].

The direct effects of Mesp2 on the suppression of Notch activity have been the subject of some debate. However, the instructive role of Mesp2 in the suppression of Notch signaling was recently demonstrated through the analysis of a knockin mouse containing a dominant-negative form of RBP-jk, the mediator of canonical Notch signaling in the *Mesp2* locus [31••]. The resulting phenotype was quite surprising since almost all of defects observed in Mesp2-null embryos were completely rescued in the DN-RBP-jk knockin mouse, indicating that most of the Mesp2 functions are mediated by the suppression of Notch activity. However, since *Mesp1*, a related gene, also has substantially similar functions to Mesp2 [32,33], the suppression of Tbx6 might be mediated by Mesp1 in these rescued embryos. A subsequent study using a cultured cell system indicated that Mesp2 suppresses Notch activity by destabilizing Mastermind-like 1 (MamL1), one of the core components of the nuclear NICD complex (Figure 8 [31••]). Surprisingly, the destabilization of MamL1 is triggered by a mutant-Mesp2 lacking the bHLH domain, indicating that Mesp2 has functions in addition to its role as a transcription factor. Thus, the basic mechanism for somite patterning is the suppression of Notch activity via Mesp2 in the rostral compartment (Figure 3b). The manner in which the Mesp2 expression domain is restricted in the rostral compartment is therefore a critical and as yet unanswered question. In a previous computer simulation study [29], Mesp2 expression was found to be dependent on Notch signal oscillation and Notch activity was observed to accumulate in the rostral compartment of the PSM (S-1) due to a slowdown in the clock oscillation speed in the embryo. This prediction is supported by the results of mutant embryo analyses such as those in the L-fng mutant (the Notch signal is always active), in which Mesp2 expression is not restricted to the rostral compartment.

Coordination of the mechanisms underlying segmental border formation and somite patterning

The Mesp2 function is closely involved in both the mechanism that leads to segmental border formation and that which underlies somite patterning. The nature of how these events are coordinated during somitogenesis is therefore an important issue. Notch and Tbx6 are upstream factors required for the activation of Mesp2. Once Mesp2 is expressed in the S-1 domain, however, it is expected to lead to MamL1 destabilization since no transcriptional activation of downstream targets is required. Thus, the suppression of Notch activity might be the immediate event that occurs upon Mesp2 expression. Mesp2 also activates its target genes *Ripply2*, by which the next segmental border is created via the suppression of Tbx6 expression, and *EphA4* which leads to the generation of the morphological border. On the basis of this scenario, rostro-caudal patterning will be established before segmental border formation. However, the extent to which suppression of Notch signaling impacts gene expression in this context is currently unknown. Further studies are warranted to assess this hypothesis. The predicted molecular cascade involved in the transition from S-1 to S0 and the establishment of rostral-caudal patterning is shown in Figure 3. Since several negative feedback mechanisms are involved in these cascades, some timing conflicts may exist. If Mesp2 expression depends only on Notch signaling, it will be downregulated when it is turned on since it induces MamL1 destabilization. However, Mesp2 expression is weakly observed even in the RBP-jk-null embryo, indicating that Tbx6 alone or other pathways might be involved in the regulation of this transcription factor. Hence, as long as the Mesp2 protein exists, the pathways leading to segment border formation and somite patterning may be active.

Perspectives

The molecular identification of the Notch oscillator and its link with the FGF signaling pathway further validated the clock and wavefront model that was proposed in 1976. However, there remain many unanswered questions regarding the precise regulatory mechanisms that underlie this process. These include the nature of why Notch oscillation ceases in the Mesp2 activation domain and whether this is due to the downregulation of Notch activity via MamL1 suppression. In this regard, it is noteworthy that Notch activity appears to continue in the absence of Mesp2. However, this is caused by Dll1 up-regulation due to the failure of Tbx6 suppression. In the wild-type mouse embryo, Notch activation is maintained in the caudal half of the somite even in the absence of Tbx6 and this is due to a positive feedback mechanism. Namely, the maintenance mechanism for Notch activity in the caudal half of the somite after segmentation differs from that of Notch oscillation observed in the PSM. It is clear therefore that something changes after Mesp2 expression but the mechanism involved is not yet known. It may be reasonable to expect that some epigenetic changes at the chromatin occur at this turning point, although no such modifications have been reported to date.

The pathway involved in MamL1 destabilization is also not yet known. Similarly, the mechanism of Tbx6 degradation has not been clarified yet, although Ripply is implicated in this suppression pathway [22•,34]. Ripply is also known to work as a modifier of Tbx6 activity in

zebrafish [35], indicating some functional differences among species. Finally, it should be noted that we have very limited knowledge of the post-translational regulation events that take place during these processes. Since transcriptional regulation alone cannot account for the observed negative feedback mechanisms, most of the proteins that function in these pathways must be under strict post-translational control during somitogenesis. Some as yet unidentified molecules that are involved in these processes must also exist. It will be important to screen for novel factors that function downstream of Mesp2 and also for partner proteins of Mesp2 or Ripply2.

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- •• of outstanding interest

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