The oscillation of Notch activation, but not its boundary, is required for somite border formation and rostral-caudal patterning within a somite

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SUMMARY
Notch signaling exerts multiple roles during different steps of mouse somitogenesis. We have previously shown that segmental boundaries are formed at the interface of the Notch activity boundary, suggesting the importance of the Notch on/off state for boundary formation. However, a recent study has shown that mouse embryos expressing Notch-intracellular domain (NICD) throughout the presomitic mesoderm (PSM) can still form more than ten somites, indicating that the NICD on/off state is dispensable for boundary formation. To clarify this discrepancy in our current study, we created a transgenic mouse lacking NICD boundaries in the anterior PSM but retaining Notch signal oscillation in the posterior PSM by manipulating the expression pattern of a Notch modulator, lunatic fringe. In this mouse, clearly segmented somites are continuously generated, indicating that the NICD on/off state is unnecessary for somite boundary formation. Surprisingly, this mouse also showed a normal rostral-caudal compartment within a somite, conferred by a normal Mesp2 expression pattern with a rostral-caudal gradient. To explore the establishment of normal Mesp2 expression, we performed computer simulations, which revealed that oscillating Notch signaling induces not only the periodic activation of Mesp2 but also a rostral-caudal gradient of Mesp2 in the absence of striped Notch activity in the anterior PSM. In conclusion, we propose a novel function of Notch signaling, in which a progressive oscillating wave of Notch activity is translated into the rostral-caudal polarity of a somite by regulating Mesp2 expression in the anterior PSM. This indicates that the initial somite pattern can be defined as a direct output of the segmentation clock.

KEY WORDS: Notch signaling, Hes7, Mesp2, Segmentation clock, Presomitic mesoderm, Lunatic fringe, Somitogenesis

INTRODUCTION
The periodicity of the segmented somites is established in the posterior presomitic mesoderm (PSM) via the function of a so-called molecular clock, which is based on complex gene regulatory networks under the control of three major signaling pathways: Notch, Fgf and Wnt (Dequeant et al., 2006; Dequeant and Pourquie, 2008). Among these pathways, Fgf and Wnt are implicated in the establishment of normal Mesp2 expression, whereas Notch signaling might be directly involved in the rostral-caudal compartmentalization within a somite. This suggests that the initial somite pattern can be defined as a direct output of the segmentation clock.

The oscillation of Notch activation, but not its boundary, is required for somite border formation. The oscillation of Notch activity is translated into the rostral-caudal polarity of a somite by regulating Mesp2 expression in the anterior PSM. This indicates that the initial somite pattern can be defined as a direct output of the segmentation clock.

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Accepted 17 February 2010
posterior PSM. The resulting transgenic mouse shows no clear cNICD on/off state in the anterior PSM. Nevertheless, this mouse exhibits normal boundary formation, indicating that the cNICD boundary is dispensable for somite formation. In addition, our transgenic mouse shows normal R-C patterning within a somite. Further analyses by computer simulation have led us to conclude that Notch signaling oscillation functions as an output signal that is both required and sufficient to establish the Mesp2 expression pattern needed for normal somitogenesis.

**MATERIALS AND METHODS**

**Animals**

The wild-type mice used in this study were the MCH strain (a closed colony established at CLEA, Japan). The Lfng-null (Evvard et al., 1998), Mesp2-null (Mesp2MCat) (Takahashi et al., 2007) and Mesp2-lacZ (Mesp2lacZ) (Takahashi et al., 2000) mouse lines are maintained in the animal facility of the National Institute of Genetics and National Institute of Health Sciences, Japan.

**Gene targeting strategy to generate the Mesp2^{Nfg} allele**

The knock-in strategy used to target the Mesp2 locus is largely similar to our previously described method (Takahashi et al., 2000), except that Lfng cDNA was inserted. The pgk-neo cassette flanked by a lox sequence was removed by crossing with CAG-Cre mice (Sakai and Miyazaki, 1997).

**Generation of the Hes7-Lfng transgenic mice**

We used a 12 kb Hes7 gene cassette comprising 5 kb of upstream sequence and all of the exons and introns, as this construct had previously been confirmed to be sufficient to reproduce the endogenous Hes7 oscillation pattern when inserted in-frame at the translational start site (Kageyama et al., personal communications). We generated the construct Lfng IRES-EGFP, in which IRES (internal ribosomal entry site)-EGFP (enhanced GFP) was fused to the 3' end of Lfng cDNA, and inserted this construct into the Hes7-translational initiation site. The resulting DNA was digested with restriction enzymes to remove vector sequences and gel purified. Transgenic mice were generated by microinjection of this construct into fertilized eggs, which were then transferred into the oviducts of pseudopregnant foster females.

**In situ hybridization, immunohistochemistry, histology and skeletal preparations**

The methods used for wholemount in situ hybridization, section in situ hybridization, immunohistochemistry, histology and skeletal preparation by Alcian Blue/Alizarin Red staining are described in our previous reports (Morimoto et al., 2005; Ogimura et al., 2008; Takahashi et al., 2000). The cNICD signal was detected by immunohistochemistry using anti-cleaved cNICD signal was detected by immunohistochemistry using anti-cleaved cNICD antibody (Val1744; 1:500; Cell Signaling Technology). Probes were prepared by PCR-amplification of GFP cDNA.

**Computer simulation**

Our computer simulation model is based on the previous mathematical description of a clock-and-wavefront model constructed by J. Lewis (Palermo et al., 1997). By using the basic oscillating function in the Lewis model, we modeled the activity of cNICD, n, at given time, t, and anteroposterior position, x, as:

\[
m(x,t) = \left[1 - \cos \left(2\pi \frac{t}{t_{r} + 1 + e^{-(x-x_{0})^2}}\right)\right]/2.
\]

For the control simulation with constant activity of cNICD, the cNICD activity, n, was set to 0.3. For the simulation with oscillating cNICD without wave, n was formulated as \(n(x)=1-\cos(\pi x)/2\). The activity of Fgf8 is known to gradually decrease from posterior to anterior, and also according to the time elapsed. These features of Fgf8 fit well with the formulation of the clock cycling rate in the Lewis model and, thus, we calculated the activity of Fgf8, f, using the formula \(f(x,t)=1/(1+e^{-c_{Fgf}^2})\).

We next added the regulation of Mesp2 and Tbx6 expression to the model. As cNICD and Fgf8 play positive and negative roles for Mesp2 expression, respectively, we assumed that the increase of Mesp2 expression occurs when the cNICD activity, n, exceeds that of Fgf8, f, with the amount dependent on n-f. Tbx6 (b) is also required for Mesp2 expression. We thus modeled the Mesp2 mRNA expression, m, and Mesp2 protein expression, p, as:

\[
m(x,t+\Delta t) = m(x,t) + S_p \times \frac{[m(x,t) - f(x,t)] / K_p^m}{1 + [m(x,t) - f(x,t)] / K_p^m} \\
+ \frac{b(x,t) / K_b^m}{1 + b(x,t) / K_b^m} - D_m \times m(x,t),
\]

\[
p(x,t+\Delta t) = p(x,t) + S_p \times (m(x,t-T) - D_p \times p(x,t),
\]

with the initial condition \(m(x,0)=0\), and \(p(x,0)=0\). The degradation of Tbx6 is dependent on Mesp2 (Ogimura et al., 2008). We introduced a hypothetical molecule, z, that is expressed depending on Mesp2 and degrades Tbx6 by interacting with it. The expression of Tbx6 (b) and the Tbx6 degrading molecule (c) were modeled as:

\[
z(x,t+\Delta t) = z(x,t) + S_p \times \frac{p(x,t-T) / K_p^z}{1 + p(x,t-T) / K_p^z} - D_z \times z(x,t) \\
+ b(x,t) / K_b^z - \frac{b(x,t) / K_b^z}{1 + b(x,t) / K_b^z} \times (z(x,t) / K_b^z),
\]

with the initial condition \(z(x,0)=0\), and \(b(x,0)=1.0\).

These formulas were implemented using C language. The activities of cNICD (n), Fgf8 (f), Mesp2 (m and p), Tbx6 regulator (z) and Tbx6 (b) were calculated over the ranges \([-12.5, 2.5]\) and \(0\) or \(2.5\). The calculations were conducted discretely with a single unit of \(t\) (\(\Delta t\)) of 1/10 and \(t\) (\(\Delta t\)) of 1/10. The parameter values we used are shown in Table S1 in the supplementary material. We also introduced time delay, \(T=2\Delta t\), for protein expression (Lewis, 2003), which did not affect the results much.

**RESULTS**

**Dissection of the Lfng expression pattern in the PSM**

To examine the significance of the Notch on/off state during boundary formation, we focused on Lfng expression, which exhibits a biphasic pattern involving oscillation in the posterior PSM and a stabilized striped pattern in the anterior PSM (Aulehla and Johnson, 1999; Cole et al., 2002; McGrew et al., 1998; Morales et al., 2002). Each of these two patterns is implicated in the generation of the corresponding Notch activity profile via negative regulation. To induce only the oscillatory expression of \(Lfng\), we utilized the Hes7 transcriptional regulatory unit as the oscillation of \(Lfng\) and Hes7 is regulated by similar factors, i.e. positively by Notch signaling and negatively by Hes7 protein. As shown in Fig. 1, these two transcripts show similar expression patterns in the oscillation phase. Both signals manifest a wavy pattern within the Tbx6 expression domain from phase I to phase III (Fig. 1A-L). However, in phases I-II, Hes7 expression is lost from the anterior domain (Fig. 1G-J), whereas that of Lfng persists for a longer period in the anterior PSM and forms a clear stripe (Fig. 1A-D,M,O). It should also be noted that the anterior Lfng expression domain was found to merge with that of the Mesp2 protein (Fig. 1N,P), the expression of which is restricted to the anterior PSM. This is not unexpected as Lfng expression is induced by Mesp2 in the anterior PSM and creates the Notch on/off state (Morimoto et al., 2005). Taken together, we concluded from these data that the Lfng expression pattern can be reproduced by two distinct regulatory systems – the Hes7 promoter-enhancer and the Mesp2 regulatory system – and this enabled us to further investigate the significance of Notch activities.
The cNICD on/off state is not required for somite boundary formation

To further elucidate the functional significance of the oscillatory cNICD in the posterior PSM and that of the cNICD on/off state in the anterior PSM, we generated a transgenic mouse line by inserting Lfng cDNA flanked with IRES-EGFP under the control of the Hes7 promoter (see Fig. S1A in the supplementary material). As expected, the expression pattern of this transgene, examined by in situ hybridization using EGFP as a probe, was found to be very similar to that of endogenous Hes7 and Lfng except for the lack of anterior striped expression (see Fig. S1B-D in the supplementary material).

We then introduced this transgene into the Lfng-null genetic background to establish the Hes7>Lfng+/Lfng–/– mouse line and examined the expression pattern of exogenous Lfng and cNICD expression in the absence of endogenous Lfng expression (i.e. an Lfng-null background). In wild-type embryos, Lfng and cNICD showed biphasic patterns, these being oscillation in the posterior PSM and stabilization in the anterior PSM, whereas cNICD oscillation was barely detectable and a constant level of cNICD could be observed through the entire PSM in the absence of Lfng, as reported previously (Morimoto et al., 2005). In the Hes7>Lfng+/Lfng–/– embryo, however, we observed the recovery of cNICD oscillation in the posterior PSM, which overlapped with Lfng expression (Fig. 2A-F), clearly indicating that the Lfng transgene was functionally active in these embryos. In addition, we previously showed that cNICD and Mesp2 generate a clear boundary in the anterior PSM, which demarcates the presumptive segmental border in phase-II embryos (Morimoto et al., 2005) (Fig. 2G-I). In the absence of Lfng, this clear border between cNICD and Mesp2 was not generated and a merged pattern was instead observed.
In the Hes7>Lfng/Lfng–/– embryo, as expected by the lack of Lfng expression in the anterior PSM, we did not detect segregation between the cNICD and Mesp2 domains (Fig. 2M-O). Lfng–/– embryos did not show clear somite boundaries, although incomplete somites did appear to be formed (see Fig. S2 in the supplementary material), as also suggested previously (Evrard et al., 1998; Zhang and Gridley, 1998). Very surprisingly, however, Hes7>Lfng/Lfng–/– embryos showed clearly segmented somites (Fig. 3A-C). This strongly indicates that the oscillatory expression of cNICD mediated via oscillating Lfng is sufficient to provide the conditions for normal somitogenesis to occur and that the cNICD boundary in the anterior PSM is not required for this process.

Recently, we and others have suggested that the Mesp2 downstream events, such as the activation of ephrin-EphA4 signaling and the formation of a Tbx6 protein boundary, were more important for segmental border formation (Watanabe et al., 2009; Oginuma et al., 2008; Nakajima et al., 2006). In Lfng–/– embryos, the expression of EphA4 and the Tbx6 protein boundary were found to be diffuse or randomized (Fig. 3E-J-L), whereas in Hes7>Lfng/Lfng–/– embryos, these expression patterns appeared to be normal (Fig. 3F,M-O), i.e. similar to those in wild-type embryos (Fig. 3D,G-I). Taken together, our current findings show that the cNICD boundary is dispensable, but that the Mesp2 boundary might be required, for the creation of the segmental border through the regulation of downstream genes.

**R-C polarity is completely recovered in Hes7>Lfng/Lfng–/– embryos**

We next further examined the morphological features of the Hes7>Lfng/Lfng–/– embryo. Surprisingly, these transgenic embryos showed a completely normal skeletal system, with segmented vertebra and ribs (Fig. 4A-C). Furthermore, the expression pattern of Uncx4.1, a caudal marker of R-C polarity (Fig. 4D), was fully recovered in the Hes7>Lfng/Lfng–/– embryo (Fig. 4F), which contrasts with the randomized pattern we observed in the Lfng–/– embryo (Fig. 4E). These results suggest that the cNICD boundary in...
the anterior PSM is not required for normal R-C polarity patterning. To elucidate this issue further, we focused on the expression of Mesp2, which is thought to be the final output signal of the segmentation clock. Mesp2 is initially expressed over one somite length and then becomes localized in the rostral compartment (Takahashi et al., 2000). This dynamic expression pattern generates a gradient of Mesp2 activity that allows PSM cells to form the R-C pattern within a somite (Takahashi et al., 2003; Takahashi et al., 2000). We therefore next compared the Mesp2 expression pattern at the cellular level among the wild-type, Lfng−/− and Hes7> Lfng/Lfng−/− embryos using high-resolution in situ hybridization. By focusing on the length of the Mesp2 transcription domain along the A-P axis, we found four distinct patterns in the wild-type embryos: (1) no signal (n=4/15); (2) most cells show nuclear dots indicating transcriptional initiation, and the length of the Mesp2 transcription domain is approximately 11-13 cells (Fig. 4G; n=3/15); (3) active stage in which signals can be observed in the cytoplasm in addition to nuclear dots, and the length of Mesp2 transcription is approximately 10-12 cells, with anterior cells showing stronger signals (Fig. 4H; n=5/15); and (4) rostral localization in which the length of the Mesp2 transcription domain becomes approximately 5-6 cells (Fig. 4I; n=3/15). In contrast to wild-type embryos, only one pattern was observed in the Lfng-null embryos: signals were observed in the cytoplasm in addition to nuclear dots, the expression levels were randomized for each cell, and the length of the Mesp2 transcription domain was approximately 9-11 cells (Fig. 4J; n=11/11). These results indicate that Mesp2 expression is always present in the anterior PSM without clear on/off cycles in the Lfng-null embryo. In addition, the Mesp2 expression domain is kept to one somite length and there is no clear localization into the rostral compartment, although cellular or cell cluster-level localization might occur in a salt-and-pepper pattern in the absence of Lfng. Importantly, the Mesp2 expression pattern was found to show four distinct patterns similar to those in wild-type embryos even in the Hes7> Lfng/Lfng−/− embryos, i.e. no signal (1/9), transcriptional initiation (Fig. 4K, n=2/9), active stage (Fig. 4L, n=3/9) and rostral localization (Fig. 4M, n=3/9). Our findings thus indicate that the oscillation of cNICD alone is sufficient to generate the normal Mesp2 expression pattern and that the anterior PSM-specific regulation of cNICD via Lfng is dispensable for this process.

**Modeling of the Mesp2 expression pattern**

To test the validity of our above hypothesis, we performed computer simulations. Our model is based on that previously proposed by Lewis and colleagues, in which the oscillatory waves emanate, travel and eventually cease, as it adopts the notion of maturity, which delays the oscillation cycle towards the anterior as time proceeds (Palmeirim et al., 1997). In this model of Lewis, the cessation of the oscillatory waves triggers periodic gene expression along an anterior-posterior direction that leads to the formation of the somites (Palmeirim et al., 1997). In our current study, we applied the Lewis model to the oscillatory waves of the cNICD and assumed Cgf as a molecular basis for maturity. We further incorporated the regulatory network required for Mesp2 expression, in which cNICD oscillation and Tbx6 synergistically activate (Yasuhioko et al., 2006; Oginuma et al., 2008), whereas the Cgf gradient suppresses Mesp2 expression and Tbx6 is degraded downstream of Mesp2 (Fig. 5A). Very surprisingly, this simple simulation successfully mimicked some specific features of dynamic Mesp2 transcription (red line), not only in terms of on/off cycles but also with regard to temporal changes in the expression pattern (from one somite length to rostral localization) along the anterior-posterior axis (Fig. 5B; see Movie 1 in the supplementary material), similar to that observed in vivo (Fig. 4G-I; see Fig. S3A,B in the supplementary material). In addition, this simulation also reproduced the gradient of Mesp2 activity accumulation (black line), which is similar to the Mesp2-β-gal pattern we observed in the Mesp2Δa2β−/− embryos (see Fig. S3E in the supplementary material).

To test the importance of the cNICD wave for the gradient formation of Mesp2 activity, we examined Mesp2 expression under constant activity of cNICD in the PSM (Fig. 5C; see Movie 2 in the supplementary material). In this instance, Mesp2 expression is always observed in the anterior PSM without either clear on/off cycles or localization at the rostral compartment, which is very similar to the in vivo situation of the Lfng−/− embryos (Fig. 4J; see Fig. S3C,D,F in the supplementary material). Interestingly, in our model, neither the formation of the waved pattern of cNICD nor its migration is necessary to establish the gradient of Mesp2 activity because a spatially uniform, but temporally oscillating, Notch signaling activity is sufficient to reproduce this gradient (see Fig. S4 and Movie 3 in the supplementary material). Without a traveling wave, however, the temporal transition of the Mesp2 expression pattern from a one-somite length to a rostral localization was not reproduced (see Fig. S4 and Movie 3 in the supplementary material). We thus speculate that this transition might be important for robust somite formation with a correct R-C polarity and propose that the wave of Notch activity enables PSM cells to establish not only the periodic expression of Mesp2, but also their localization into the rostral compartment. Our model therefore provides a new concept that indicates that a progressive oscillating wave of Notch activity is translated into the R-C polarity of a somite through the regulation of the Mesp2 expression pattern.

**Anterior PSM-specific Lfng cannot rescue the defects in Mesp2-null or Lfng-null mice**

Finally, to further ask the significance of the anterior striped cNICD domain for somite boundary formation, we established a mouse line resulting heterozygous mice showed no abnormalities and we generated an intercross of Mesp2Lfng/Lfng mice. The expression of cNICD via Lfng is dispensable for this process. In the Mesp2Lfng/Lfng embryos, cNICD signals were suppressed in the Lfng-expressing cells in the anterior PSM (Fig. 6B), as seen in the wild type (Fig. 6A). We further found that some of the cells that did not express Lfng maintained cNICD signals, indicating that Lfng suppresses cNICD production in a cell-autonomous manner (Fig. 6D). However, Lfng did not rescue the phenotype of the Mesp2-null mice (Fig. 6H-M), indicating that the function of Lfng downstream of Mesp2 is not important.

We next introduced this transgene into the Lfng-null genetic background to generate a Mesp2Δa2Δcgf- Lfng−/− mouse. The expression levels of Lfng in the Mesp2 locus were found to be low (Fig. 6C,E; see Fig. S5C in the supplementary material), but we did observe downregulation of the cNICD signal in the Mesp2-expressing cells (Fig. 6F) in comparison with the Lfng-null embryos (Fig. 6G). Furthermore, Hes5 expression (see Fig. SSD in the supplementary material), a target gene of Notch signaling, was severely downregulated in both the Mesp2Lfng/Lfng and Mesp2Δa2Δcgf-Lfng−/− embryos (see Fig. S5F,H in the supplementary material) compared with Mesp2- and Lfng-null embryos (see Fig. S5E,G in the supplementary material), indicating that Lfng under the control of Mesp2 might effectively suppress Notch signaling. However, we did
not detect any significant rescue of the segmental morphology in the developing embryos or of the vertebral morphology at any level along the anteroposterior axis in the developing embryos or of the vertebral morphology at any level.

**DISCUSSION**

**The requirement for Notch signaling during mouse somitogenesis**

In our current study, we reveal that the cNICD on/off state is not required for somite boundary formation during somitogenesis in the mouse. Consistent with this, recent studies in zebrafish embryos suggest that the function of Notch signaling is only to synchronize the oscillations among PSM cells, and that this pathway has no other function during segmentation (Riedel-Kruse et al., 2007; Horikawa et al., 2006; Ozbudak and Lewis, 2008). However, we propose from our current data that Notch signaling has a crucial function also as an output of the segmentation clock during mouse development.

This contention is supported by earlier evidence that *Mesp2* expression is severely downregulated in the absence of Notch signaling (Barrantes et al., 1999; Takahashi et al., 2000). Moreover, it has been shown that constitutive activation of Notch signaling in the paraxial mesoderm induces *Mesp2* transcription without clear on/off cycles (Feller et al., 2008) and it is also evident from other reports that Notch signaling is crucial for the establishment of R-C patterning of somites in the mouse (Takahashi et al., 2000; Takahashi et al., 2003; Feller et al., 2008). These results together suggest that the function of Notch signaling is not only to synchronize oscillations but that Notch acts also as an important output signal of the segmentation clock, at least in mouse somitogenesis. We thus speculate that Notch signaling is a key factor that mediates the transduction of clock activities into the morphological segmental pattern by regulating *Mesp2* expression.

**A new model for the establishment of R-C polarity during somitogenesis**

Based on our present findings, we propose a new function for oscillating Notch signaling, which is translated into the R-C polarity of a somite via the regulation of *Mesp2* expression in the anterior PSM. Previous models have proposed that the establishment of R-C polarity requires cell-cell communication (Takahashi et al., 2003; Dale and Pourquie, 2000), whereas we propose a model in which a
cell-autonomous mechanism utilizes Notch signaling oscillation in the posterior PSM. This notion is further supported by computer simulations, in which we found that an appropriate translation of spatiotemporal information provided by the traveling wave of cNICD is sufficient to create the dynamic Mesp2 expression pattern, i.e. on/off cycles and rostral localization (Fig. 5B; see Movie 1 in the supplementary material). In these simulation experiments, the generation of the traveling wave was based on the earlier work of Lewis (Palmeirim et al., 1997), and the translation of the wave information into Mesp2 expression was modeled on the gene network that we elucidated previously (Oginuma et al., 2008; Yasuhiko et al., 2006). In the model, the cNICD wave, an activator of Mesp2, travels from the posterior to the anterior, whereas the levels of Fgf, a repressor of Mesp2, are higher toward the posterior. Consequently, as a single wave passes through a nascent somite, the net transcriptional activation of Mesp2, which reflects the amount of cNICD subtracted by the amount of Fgf, is higher toward the rostral part of the presumptive somite. The resulting gradient of Mesp2 activity might thus allow PSM cells to establish a rostral identity and the segmental border. Hence, this is the first model to demonstrate that R-C polarity in the somite is generated as a direct output of the segmentation clock.

The repression of Tbx6, an activator of Mesp2, downstream of Mesp2 is another important component in our model. This regulatory module prevents Mesp2 expression after one traveling wave of cNICD has passed, and thus fixes the R-C gradient pattern of Mesp2. The next wave of Notch signaling cannot affect the Mesp2 pattern created by the former wave. To reproduce the intensive degradation of Tbx6 at anterior regions, we had to adjust the parameters for Tbx6 degradation. We did not need to change any of the other standard parameters we initially chose, suggesting that the qualitative features of the model are not so sensitive to the quantitative values of the parameters. In our simulation analysis, however, we did not reproduce the sharp anterior boundaries of Tbx6 and Mesp2 accumulation (green and black lines, respectively, in Fig. 5B; see Movie 1 in the supplementary material) that have been observed in vivo. To create a sharp boundary of Tbx6 and Mesp2, which should be required to create a fine segmentation boundary, further adjustment of the parameters or another mechanism might be required. In this regard, the next important challenge will be to investigate the molecular basis of the sharpening expression boundaries of Tbx6 and Mesp2, and ultimately to understand how analog inputs (such as sequential wave patterns of oscillation) are converted into digital outputs (such as the square-like stair patterns of the segmental border).

Functions of Lfng in the posterior and anterior PSM during mouse somitogenesis

We also demonstrate from our present data that the oscillatory expression of Lfng is both required and sufficient for normal somitogenesis. However, this result will probably be viewed somewhat controversially given the recent findings that have underscored the significance of Lfng expression in the anterior PSM during this process, at least after E10.5 (Shifley et al., 2008; Stauber et al., 2009). The authors of these reports produced transgenic mice harboring Lfng expression without oscillation. Their data indicate that cNICD oscillation is disrupted, but that normal segmented somites form, after E10.5 and they concluded that oscillating Lfng expression is required only for early stage, but not later stage, somitogenesis (Shifley et al., 2008; Stauber et al., 2009). We wish therefore to discuss some possible explanations for the discrepancies between our current findings and these previous experimental results.

One possibility is that the common expression profiles between our Hes7>Lfng mouse and the mice studied in previous reports is important. We demonstrate here that Hes7 and Lfng expression manifest a waved pattern within the Tbx6 expression domain, which includes a part of the anterior PSM. Therefore, in our Hes7>Lfng mouse, oscillating Lfng expression also exists in the anterior PSM, but not as a stabilized pattern. We suspect that the transgenic mice analyzed in previous reports lack oscillating Lfng expression in the posterior PSM but the oscillation might exist in the anterior PSM as well, and thus we speculate that the oscillating Lfng expression in the posterior PSM is not required after E10.5, but that in the anterior PSM might be sufficient for normal somitogenesis. Another possibility is that the slightly oscillating expression reported previously might be responsible for the rescue event. Previous studies have shown that two distinct enhancers are involved in the oscillatory expression of Lfng, one of which is disrupted in the mouse reported by Shifley et al. (Shifley et al., 2008), and a slight cyclic expression of Lfng exists in the mouse generated by Stauber et al. (Stauber et al., 2009). Hence, one possible interpretation for these discrepancies is that the slight cyclic expression of Lfng might be sufficient for normal development in the enhancer-specific
knockout mouse after E10.5 somitogenesis, but not prior to E10.5. It is reasonable to assume that the requirement of Notch clock oscillation by Lfng changes during somitogenesis and is lesser at later stages, as now suggested by a number of studies (Shifley et al., 2008; Stauber et al., 2009).

Acknowledgements

We thank Ryoichi Kageyama (Kyoto University) for providing the Hes7 promoter and enhancer cassette, Aya Satoh, Nobuo Sasaki and Yusuke Okubo (National Institute of Genetics) for animal care and the preparation of embryo samples and Maniko Ikumi, Eniko Ikeno and Shinobu Watanabe (National Institute of Genetics) for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas, Dynamics of Extracellular Environments, and by the National BioResource Project from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.044545/-/DC1

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