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Analysis of Ripply1/2-deficient mouse embryos reveals a mechanism underlying the rostro-caudal patterning within a somite

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ABSTRACT

The rostro-caudal patterning within a somite is periodically established in the presomitic mesoderm (PSM). In the mouse, Mesp2 is required for the rostral property whereas Notch signaling and Ripply2, a Mesp2-induced protein that suppresses *Mesp2* transcription, are required for the caudal property. Here, we examined the mechanism behind rostro-caudal patterning by comparing the spatial movement of Notch activity with Mesp2 protein localization in wild-type embryos and those defective in *Ripply1* and *2*, both of which are expressed in the PSM. Mesp2 protein appears first as a thin band in the middle of the traveling Notch active domain in both wild-type and *Ripply1/2*-deficient embryos. In wild-type embryos, the Mesp2 band expands anteriorly to the expression front of Tbx6, an activator of *Mesp2* transcription. Notch activity becomes localized further anteriorly to this Mesp2 domain, but does not pass over the anterior Mesp2 domain generated in the previous segmentation cycle. As a result, the Notch active domain appears to be restricted between these two Mesp2 domains. In *Ripply1/2*-deficient embryos, the Mesp2 band becomes more expanded and the Notch domain is finally diminished. Interestingly, *Ripply1/2*-deficient embryos exhibit anterior expansion of the Tbx6 protein domain, suggesting that Ripply1/2 regulates *Mesp2* expression by modulating elimination of Tbx6 proteins. We propose that the rostro-caudal pattern is established by dynamic interaction of Notch activity with two Mesp2 domains, which are defined in successive segmentation cycles by Notch, Tbx6 and Ripply1/2.

Introduction

The spatial characteristics of somites—e.g., segmental borders and the rostro-caudal pattern—are established in the anterior region of the presomitic mesoderm (PSM) at regular time intervals. This periodicity is originally generated by the so-called "segmentation clock," which manifests itself by the oscillation of gene expression (Gajewski et al., 2003; Henry et al., 2002; Hirata et al., 2002; Holley et al., 2000; Jiang et al., 2000; Oates and Ho, 2002; Palmeirim et al., 1997). In the PSM of the mouse embryo, oscillatory transcription of *Hes7*, encoding a bHLH transcription factor whose expression is induced by Notch signaling, and of *Lunatic fringe (Lfng)*, encoding a modulator of Notch signaling, travels in a posterior-to-anterior direction during each segmentation cycle (Aulehla and Johnson, 1999; Bessho et al., 2001, 2003). Notch activity itself also oscillates and travels in a similar manner (Huppert et al., 2005; Morimoto et al., 2005). This Notch oscillation is finally stabilized in the anterior PSM, where the temporal periodicity is translated into the spatial pattern that is defined by segmental borders and rostro-caudal compartments.

In this tempo-spatial transition, border formation between somites is a relatively well-characterized event. First, the presumptive somite borders are primarily defined by the "determination front," which is thought to be established in a manner dependent on the antagonistic interaction between FGF and retinoic acid (RA)-signaling gradients (Delfini et al., 2005; Diez del Corral et al., 2003; Dubrulle et al., 2001; Moreno and Kintner, 2004; Sawada et al., 2001; Wahl et al., 2007). At the determination front, expression of *Mesp2*, a bHLH transcription factor whose expression is suppressed by FGF signaling in the posterior PSM, is turned on (Delfini et al., 2005; Oginuma et al., 2008; Saga et al., 1997). During a segmentation cycle, *Mesp2* mRNA is maximally expressed in a one-somite-length fashion, then gradually contracting to the rostral half, and finally disappearing. *Mesp2* expression is dependent on the Tbx6 transcription factor and Notch signaling (Yasuhiko et al., 2006). Thus, *Mesp2* is expressed to the anterior top of the Tbx6 expression domain, which is consistent with a segmental border, and is expressed in a periodical fashion, which is defined by the

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Notch wave from the posterior PSM. On the other hand, Mesp2 leads to degradation of Tbx6 proteins in a ubiquitin-dependent manner (Oginuma et al., 2008). This degradation subsequently creates a next segmental border, or the new anterior border of Tbx6 proteins, which defines another *Mesp2* expression domain induced in the next segmentation cycle. By these coordinated interactions, the segment border is dynamically established at regular time intervals.

In addition to border formation, Notch signaling and Mesp2 activity also play roles in the rostro-caudal patterning of a somite. For instance, mouse embryos defective in Notch signaling, caused by knocking out *Dll1* or *Presenilin 1*, show rostralized somites, whereas those lacking Mesp2 activity exhibit caudalization of their somites (Hrab de Angelis et al., 1997; Koizumi et al., 2001; Saga et al., 1997; Takahashi et al., 2000). Consistent with their roles in rostro-caudal patterning, the domain of Notch activity and Mesp2 expression become contracted in the caudal half of S0 (the prospective somite in the most anterior PSM) and in the rostral half of S-I (the prospective somite posterior to S0), respectively (Morimoto et al., 2005). However, some critical questions for understanding the molecular mechanism of the rostro-caudal patterning remain to be elucidated. For instance, how does the Mesp2 activity become contracted into the rostral S-I? How does the Notch active domain become localized into the caudal S0? And an important question is how the intra-somitic border between rostral and caudal compartments is defined.

Recently, we and other groups showed that transcription factors of the Ripply family are also required for rostro-caudal patterning in several organisms (Chan et al., 2006; Kawamura et al., 2005; Morimoto et al., 2007). Ripply proteins suppress Tbx-mediated transcription of *Mesp* genes by recruiting the Groucho/TLE co-repressor (Kawamura et al., 2008; Kondow et al., 2007). In the mouse, *Ripply2*-null mutant embryos exhibit highly rostralized somites (Morimoto et al., 2007). On the other hand, *Mesp2* is required for *Ripply2* expression, indicating that *Mesp2* suppresses its own expression by activating Ripply2 expression. Based on the result that a Ripply2 deficiency leads to persistent expression of Mesp2 in the rostral compartment at S0, it was proposed that the persistence of Mesp2 expression leads to the suppression of the caudal characteristics (Morimoto et al., 2007). However, it seemed uncertain whether and how the persistently expressed Mesp2 in the rostral compartment suppresses Notch activity in the caudal compartment because of a lack of analysis of the dynamic process of the rostro-caudal patterning in *Ripply2*-deficient embryos. Furthermore, because an additional member of the Ripply family, *Ripply1*, is also expressed in the anterior PSM, it is also uncertain how the segmentation is disturbed when all of the Ripply activities are eliminated.

Therefore, in the present study, we eliminated all of the functions of the Ripplys by generating a *Ripply1* and *Ripply2* double-null mutant, and then examined the dynamic processes of rostro-caudal patterning by exhaustive examination of periodical changes in the location of the Notch active domain and the Mesp2 protein domain in wild-type and *Ripply*-deficient embryos at several distinct phases of the segmentation cycle. Based on our analysis, we propose a model that can explain the processes of localization of Notch activity into the caudal compartment and of positioning of the intra-somitic boundary between the rostral and caudal compartments. This model shows that the rostro-caudal pattern is not defined by a simple read-out of the segmentation clock, but rather is regulated by coordinated dynamic interactions between Notch activity and two Mesp2 domains, which are defined by the interaction among Notch, Tbx6, and Ripply1/2.

Materials and methods

In situ hybridization

Whole-mount *in situ* hybridization was performed following a standard procedure. For the *Ripply1* probe, we constructed pBluescriptIISK-Ripply1-R9ΔGA, containing a full-length cDNA with deletion of the guanine/adenine-rich region in the 3'UTR. The other probe used was described previously (Bessho et al., 2003; Kawamura et al., 2005; Takahashi et al., 2000, 2007).

Mice

The *Ripply2* and *Mesp2* null mice were made as described, respectively (Morimoto et al., 2005; Takahashi et al., 2000). *EIIa-Cre* transgenic mice were kindly provided Dr. H. Westphal (Lakso et al., 1996).

Targeted disruption and generation of Ripply1-deficient mice

Ripply1 genomic loci were isolated from CJ7 ES cells. CJ7 ES cells were electroporated with a linearized targeting vector and selected by G418-resistance and by PCR as described earlier (Takada et al., 1994). Targeted clones were further confirmed by Southern blot analysis. Hemizygous ES cells were injected into blastocysts of C57BL/6 mice to generate germ-line chimeras. Because *Ripply1* is on the X chromosome and CJ7 ES cells are derived from male mice, we adopted the Cre-loxP system to circumvent the possibility of male lethality in chimeric mice. Floxed *Ripply1* mice were then crossed with *EIIa-Cre* transgenic mice to remove a sequence containing the three exons and the *PGK-neo-FRT* cassette, resulting in generation of female *Ripply1* heterozygotes ($Ripply1^{-}/X$) for further generation of male $Ripply1^{-}/Y$ and female $Ripply1^{-}/Ripply1^{-}$ mutant mice. Genotypes were determined by PCR using the following three primers: F1 primer (5'-ACGAGTCTTCCTTTAGCTGC-3'), F2 primer (5'-GTTGGCGCCTACCGGTGGATGTGGAATGTGTG-3'), R1 primer (5'-AGTGGGAGGAGCTAGCAAGTGTCTGGGTCT-3'). Using RT-PCR with specific primers designed for the coding regions of the *Ripply1* gene, we detected no *Ripply1* transcripts in the neonatal tongue (data not shown), in which *Ripply1* is abundantly expressed (Kawamura et al., 2005).

Skeletal preparation

Skeletal preparation of newborns was performed as previously described with some modifications (Ohbayashi et al., 2002). Postpartum day 0 pups were skinned and eviscerated, fixed in a 99% ethanol solution for 3 days, and then treated in acetone for 3 days to remove the fat. Pups were stained for 2 days with a mixture of 0.15% Alcian blue and 0.1% Alizarin Red S in ethanol/acetic solution and cleaned in 1% trypsin for 6–12 h. A second cleaning was performed using 0.1% KOH for several hours or overnight at room temperature.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, embedded in OCT, and sectioned at 7 μm. Sections were immersed in unmasking solution (Vector Laboratories) and autoclaved at 105 °C for 15 min to enable antigen retrieval (Morimoto et al., 2005). Then, the sections were stained by using either anti-Mesp2 (1:500) (Morimoto et al., 2005), anti-activated Notch1 (1:250, Cell Signaling technology) or anti-Tbx6 (1:500) (White and Chapman, 2005) as the primary antibody, followed by a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:1000, Promega) as a secondary antibody and Cyanin3 tyramide or fluorescein isothiocyanate (FITC)-conjugated tyramide (Perkin Elmer) for signal detection.

Double immunostaining was carried out as described previously (Oginuma et al., 2008). Sections were incubated with anti-activated Notch1 (1:250) primary antibody after antigen retrieval, followed by incubation with Histofine (Nichirei Bioscience), and treatment with Cyanin3 tyramide. Second, for the detection of Mesp2 or Tbx6, the same sections were incubated with anti-Mesp2 (1:1000) or anti-Tbx6 (1:500) primary antibody, followed by incubation with horseradish