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Feedback loops comprising Dll1, Dll3 and Mesp2, and differential involvement of Psen1 are essential for rostrocaudal patterning of somites

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SUMMARY

Elaborate metamerism in vertebrate somitogenesis is based on segmental gene expression in the anterior presomitic mesoderm (PSM). Notch signal pathways with Notch ligands Dll1 and Dll3, and the transcription factor Mesp2 are implicated in the rostrocaudal patterning of the somite. We have previously shown that changes in the *Mesp2* expression domain from a presumptive one somite into a rostral half somite results in differential activation of two types of Notch pathways, dependent or independent of presenilin 1 (Psen1), which is a Notch signal mediator. To further refine our hypothesis, we have analyzed genetic interactions between *Dll1*, *Dll3*, *Mesp2* and *Psen1*, and elucidated the roles of Dll1- and Dll3-Notch pathways, with or without Psen1, in rostrocaudal patterning. Dll1 and Dll3 are co-expressed in the PSM and so far are considered to have partially redundant functions. We find in this study that positive and negative feedback loops comprising Dll1 and Mesp2 appear to be crucial for this patterning, and Dll3 may be required for the coordination of the Dll1-Mesp2 loop. Additionally, our epistatic analysis revealed that Mesp2 affects rostrocaudal properties more directly than Dll1 or Dll3. Finally, we find that Psen1 is involved differently in the regulation of rostral and caudal genes. Psen1 is required for Dll1-Notch signaling for activation of *Dll1*, while the Psen1-independent Dll3-Notch pathway may counteract the Psen1-dependent Dll1-Notch pathway. These observations suggest that Dll1 and Dll3 may have non-redundant, even counteracting functions. We conclude from our analyses that Mesp2 functions as a central mediator of such Notch pathways and regulates the gene expression required for rostrocaudal patterning of somites.

Supplemental data available online

Key words: Mesp2, Notch signaling, Rostrocaudal patterning, Presenilin, Somite segmentation, Mouse

INTRODUCTION

Somitogenesis is an intriguing example of metameric pattern formation in vertebrate embryos. Epithelial somites form at the anterior end of the unsegmented paraxial mesoderm, which is supplied by the primitive streak or tail bud, by a mesenchymal-epithelial conversion in a spatially and temporally coordinated manner. Each somite is subdivided into two compartments, the rostral (anterior) and caudal (posterior) halves. This rostrocaudal polarity appears to be established just prior to somite formation.

Studies in zebrafish, chick and mouse embryos have established that the Notch signaling pathway is essential for somite formation and patterning, particularly for the establishment of the rostrocaudal segment polarity (Conlon et al., 1995; Oka et al., 1995; Dornseifer et al., 1997; Hrabe de Angelis et al., 1997; Wong et al., 1997; Kusumi et al., 1998; Evrard et al., 1998; Zhang and Gridley, 1998; del Barco Barrantes et al., 1999; Takke and Campos-Ortega, 1999; Holley et al., 2000; Takahashi et al., 2000; Koizumi et al., 2001; Bessho et al., 2001) (reviewed by Saga and Takeda, 2001). In fact, many zebrafish and mouse mutants for genes encoding Notch pathway components exhibit defects in the rostrocaudal polarity of somites. The Notch signaling is closely linked to the putative molecular clock mechanism that operates in the PSM, as oscillating genes encode Notch pathway components and mutations in Notch pathway components also affect cyclic genes (Palmeirim et al., 1997; McGrew et al., 1998; Forsberg et al., 1998; Jiang et al., 2000; Holley et al., 2002; Oates and Ho, 2002). The generation of the rostrocaudal polarity is also thought to be controlled by the molecular clock. However, the precise nature of the molecular clock is not yet known at all. In zebrafish, defects in the rostrocaudal polarity are often not distinguished from defects in the molecular clock function, because most of Notch pathway mutants in zebrafish exhibit similar phenotypes. For example, zebrafish *aei*, *des* and *bea* mutant embryos commonly show a salt-and-pepper (randomized) expression pattern of the rostral- or caudal-half marker genes, instead of normal regular stripes (Jiang et al., 2000; Holley et al., 2002). This phenotype is virtually

indistinguishable from the phenotype seen in the *her1*- and *her7*- Morpholino-injected embryo, which shows disruption of cyclic gene expression (Oates and Ho, 2002). Thus, there is no available Notch pathway mutant in zebrafish that enables further analysis of the mechanism of rostrocaudal patterning separately from the molecular clock.

By contrast, Notch pathway mutants in mouse exhibit various patterns of phenotypes regarding the rostrocaudal polarity of somites. For example, in Delta-like 1 (Dll1)- and RBPjk-null embryos, somites show neither rostral nor caudal property (del Barco Barrantes et al., 1999), whereas Delta-like 3 (Dll3), lunatic fringe and Hes7-null embryos show a salt-and-pepper expression pattern of caudal marker genes (Kusumi et al., 1998; Evrard et al., 1998; Zhang and Gridley, 1998; Bessho et al., 2001). In our previous work, we have demonstrated that Mesp2-null and presenilin 1 (Psen1)-null embryos show opposite phenotypes with respect to the rostrocaudal polarity of somites (Takahashi et al., 2000). The Mesp2-null embryo exhibits caudalized somites, i.e., the somite loses the rostral-half property, and the whole somite acquires the caudal-half characteristics. The reverse is true for the Psen1-null embryo. These observations led us to some fundamental questions: what is the default state, and how do these genes cooperate to establish rostrocaudal segment polarity? In some mouse mutants, such as Dll3-null, oscillation of cyclic genes is disrupted (Dunwoodie et al., 2002). However, in Mesp2-null embryos, the rostrocaudal polarity is disrupted without affecting oscillation of cyclic genes in the posterior PSM (Nomura-Kitabayashi et al., 2002) (Y.T., unpublished). In Psen1-null embryos, oscillation of cyclic genes in the posterior PSM normally occurs, although the level of expression is reduced (Koizumi et al., 2001). Therefore Mesp2 and Psen1 serve as good tools for exploring mechanisms of the rostrocaudal patterning independent of molecular clock function.

Mesp2 is a member of the Mesp family, a group of bHLH transcription factors, which is expressed in the anterior PSM just prior to somite formation and is essential for somite boundary formation as well as formation of the rostrocaudal polarity (Saga et al., 1997; Nomura-Kitabayashi et al., 2001). We have previously observed that the rostrocaudal polarity of somites correlates well with the spatial pattern and the level of expression of the Notch ligand *Dll1*. Genetic analyses of Mesp2-null, and Psen1-null mice, and mice carrying an activated *Notch1* in the *Mesp2* locus have led us to propose a model for rostrocaudal patterning, in which two Notch pathways can be active in the anterior PSM. One is the Psen1-dependent Notch pathway for inducing expression of *Dll1*, and the other is the Psen1-independent Notch pathway for suppressing expression of *Dll1*. Mesp2 normally suppresses the *Dll1*-inducing pathway and potentiates the *Dll1*-suppressing pathway in a region corresponding to one presumptive somite. When *Mesp2* expression becomes restricted to the presumptive rostral half, expression of *Dll1* is induced in the presumptive caudal half by the Psen1-dependent Notch pathway (Takahashi et al., 2000). However, the ligands for these two Notch pathways have not yet been identified.

In both zebrafish and mouse embryos, at least two Notch ligands (DeltaC and DeltaD, and Dll1 and Dll3, respectively) are co-expressed in the PSM, and their expression domains are finally segregated into the rostral or caudal half of formed somites (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Haddon et al., 1998). These expression patterns imply that these ligands do not have merely redundant functions, but also have distinct roles in somite patterning and boundary formation. Despite a large number of studies, possible functional differences between Dll1 and Dll3 signals are not clear. Likewise, the roles of Psen1, a Notch signal mediator involved in nuclear translocation of the Notch intracellular domain (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999), during somitogenesis are not fully understood. If Psen1 were equally involved in all aspects of Notch signaling, it is puzzling that the rostrocaudal patterning defects of somites in the Psen1-null embryo are unique and different from that in any other Notch pathway mutants (Takahashi et al., 2000; Koizumi et al., 2001). Thus, to elucidate the precise requirements for Psen1 functions in somite patterning, further studies are required.

We have conducted genetic studies of the roles in rostrocaudal patterning of Dll1- and Dll3-mediated Notch signaling, the relationships between Notch signaling and Mesp2 function, and the involvement of Psen1 in Dll1- and Dll3-mediated Notch pathways. Our analysis of these genetic interactions revealed several novel findings.

(1) Dll1- and Dll3-Notch signaling and Mesp2 constitute a complex signaling network for stripe formation in the anterior PSM. Feedback loops of Dll1 and Mesp2 are essential for establishment of the rostrocaudal polarity, while Dll3 is necessary for localization and integration of expression of *Dll1* and *Mesp2*.

(2) Mesp2 can affect rostrocaudal properties more directly than Dll1 or Dll3.

(3) Psen1 is involved differently in Dll1-Notch and Dll3-Notch pathways.

(4) Dll3-Notch signaling can counteract Psen1-dependent Dll1-Notch signaling.

Based on these findings, we propose a new model for stripe formation in the anterior PSM, which is different from the previous hypothesis that rostrocaudal patterning, i.e. formation of the half-a-somite stripe pattern of gene expression, can be regarded as a result of stabilization of oscillating expression in the posterior PSM.

MATERIALS AND METHODS

Animals

The *Dll1*$^{+/lacZ}$ knock-in (Hrabe de Angelis et al., 1997), *Mesp2*$^{+/lacZ}$ knock-in (Takahashi et al., 2000), *Psen1*$^{+/-}$ (Koizumi et al., 2001) and *Dll3*$^{+/pu}$ (Kusumi et al., 1998) mice are maintained in the animal facility in National Institute of Health Sciences, Japan. Double heterozygous mice with an ICR background for each combination of genes are used to obtain the double homozygous embryos. The primer sets used for genotyping are as shown in the original papers.

Analysis of phenotypes

The methods for gene expression analysis by whole-mount in situ hybridization, histology and skeletal preparation by Alcian Blue/Alizarin Red staining are as described in previous paper (Saga et al., 1997). A strong emphasis was placed on obtaining a precise and accurate comparison of gene expression patterns and intensity

of signals between different genotypes. Littermate embryos from crosses of double-heterozygous parents were simultaneously fixed and processed for in situ hybridization. Coloring reactions in BM purple solution (Roche) were stopped at exactly the same time for each embryo. To evaluate gene expression precisely in the double mutant embryo, simultaneous staining of wild-type and single mutant littermates as controls is essential. Therefore, in all of the images presented in the figures, the arranged embryos are littermates. At least four, but more usually six, double-null embryos were used for gene expression analysis with more than ten single mutants and many more wild-type embryos. Observed differences in gene expression levels were typically reproduced in triplicate. In the case of skeletal morphologies, each of eight Dll3/Mesp2 double-null fetuses exhibited almost complete fusion of neural arches. For vertebral morphologies in Dll3/Psen1 intercrosses, the number of fetuses is presented in supplementary data S2F. Each of six Dll3/Psen1 double-null fetuses showed reduced amounts of disorganized skeletal elements. Whole-mount specimens and skeletal preparations were observed and photographed with a Leica dissection microscope equipped with a Fujifilm digital camera (HC-2500) under specific illumination conditions.

RESULTS

Positive and negative feedback loops of Dll1 and Mesp2 are essential for stripe formation

We have demonstrated that suppression of *Dll1* by Mesp2 is essential for the establishment of rostrocaudal polarity and both activation and suppression of *Dll1* are mediated by Notch signaling through ligands which have not yet been defined. To address this question, we used mouse genetics to analyze the functional relationship between Dll1 and Mesp2. First we examined auto- and reciprocal regulations of *Dll1* and *Mesp2*. As the Dll1-null embryo has a *lacZ* knock-in allele (Hrabe de Angelis et al., 1997), we can observe expression of *Dll1-lacZ* in the absence of the Dll1 function. In the $Dll1^{+/L}$ embryo, *lacZ* expression reflects the normal expression pattern of *Dll1*, showing strong staining in the PSM and stripes in the caudal halves of somites (Fig. 1A,B). Sporadic expression in the neural tube is also noted. By contrast, in the $Dll1^{L/L}$ embryos, the *Dll1-lacZ* stripes are not

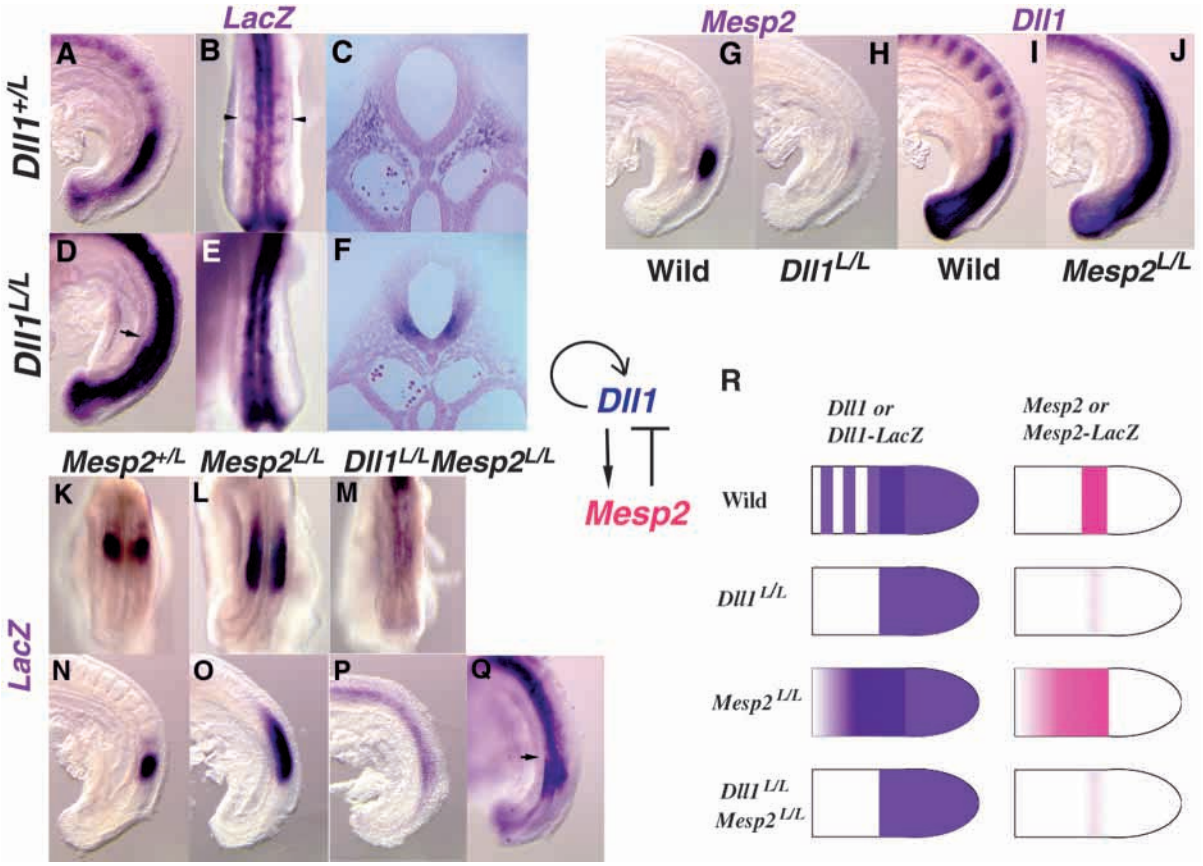


Fig. 1. Positive and negative feedback loops of Dll1 and Mesp2 are essential for stripe formation. (A-F) Dll1 induces expression of *Dll1* itself. Expression of *Dll1-lacZ* mRNA was detected by in situ hybridization in $Dll1^{+/L}$ (A-C) and $Dll1^{L/L}$ (D-F) embryos at 9.5 dpc. (A,D) Lateral view, (B,E) dorsal view of the tail region. (C,F) Transverse section at the anteriormost PSM. In the $Dll1^{+/L}$ embryo, *lacZ* expression reflects normal stripe pattern of *Dll1*, localized at the caudal half of somites (arrowheads in B). In the $Dll1^{L/L}$ embryo, the stripe of *Dll1-lacZ* is lost at the putative somite region (anterior to the arrow in D). Ectopic strong staining in the ventral neural tube is evident (F). (G-J) Expression of *Mesp2* is severely decreased in the Dll1-null embryo (G,H) while expression of *Dll1* is strongly expanded in the Mesp2-null embryo (I,J). (K-Q) *Mesp2-lacZ* mRNA (with *Dll1-lacZ* in case of the double mutant) was detected by in situ hybridization. (K-M) Dorsal views and (N-Q) lateral views. After extended staining, *Dll1-lacZ* expression appears at the neural tube and the PSM, but not at the somite region (Q, arrow indicates the putative boundary between PSM and somite region). (R) Summary of reciprocal regulation of *Dll1* and *Mesp2*. In the absence of Dll1, both *Dll1* stripes and normal level of *Mesp2* expression are lost. In the absence of Mesp2, both *Dll1* and *Mesp2-lacZ* expressions are strongly expanded. The Dll1/Mesp2 double-null embryo is similar to the Dll1-null embryo in terms of reciprocal regulation.

detected in the putative somite region, even after extended color development. Expression in the PSM appears not to be affected, but shows a sharp border in the anterior PSM (Fig. 1D,E, arrow in D). It is noteworthy that strong and uniform *lacZ* expression is observed in the ventral neural tube, suggesting the lack of lateral inhibition (Fig. 1C,F). The different effects of the loss of Dll1 on *Dll1* transcription in the neural tube and somites suggest that the *Dll1* stripe formation in the rostral PSM is not a result of the lateral inhibition, but that Dll1 function itself is required for the formation of the *Dll1* stripes. Thus, Notch ligand that induces *Dll1* expression is Dll1 itself. However, *Dll1* expression in the posterior PSM seems to be independent of Dll1-Notch signaling. The loss of *Dll1-lacZ* stripes was also observed in the Dll1/Mesp2 double-null embryo, indicating that it is independent of the Mesp2 function (Fig. 1Q).

As reported previously (del Barco Barrantes et al., 1999), the expression of *Mesp2* is severely downregulated in the Dll1-null embryo (Fig. 1G,H), while strong expression of *Dll1* is expanded in the Mesp2-null embryo (Fig. 1I,J) (Takahashi et al., 2000). These observations indicate that Dll1 induces expression of *Dll1* itself and *Mesp2*, whereas Mesp2 suppresses expression of *Dll1*. This genetic cascade may propagate via the Dll1-Notch signaling pathway, and thus this feedback loop might function at the tissue level. Moreover, this genetic cascade explains the autoregulatory nature of *Mesp2* expression. We have noticed in our previous work that expression of *Mesp2* itself (*Mesp2-lacZ*) is strongly expanded in the absence of the Mesp2 function (Fig. 1K,L,N,O). This expansion of *Mesp2-lacZ* expression is coincident with the expansion of *Dll1* expression [see figure 5 by Takahashi et al. (Takahashi et al., 2000)]. In addition, this expanded expression of *Mesp2-lacZ* is lost in the Dll1/Mesp2 double-null embryo, indicating that it is dependent on Dll1 (Fig. 1M,P). The auto- and reciprocal regulations of *Dll1* and *Mesp2* are illustrated in Fig. 1R. Thus, Dll1-Notch signaling results in both activation and suppression of *Dll1* expression.

Mesp2 affects rostrocaudal properties more directly than Dll1

Next, we analyzed interactions between *Dll1* and *Mesp2* in regulation of rostral and caudal half marker genes, to address which gene more directly specifies rostrocaudal properties. In the *Mesp2*-null embryo, expression of the rostral marker genes *Cer1* and *Notch2* is severely decreased, while expression of the caudal marker genes *Dll1* and *Uncx4.1* is strongly expanded, suggesting that Mesp2 suppresses caudal and activates rostral properties. However, expression of both rostral and caudal marker genes is severely decreased in the Dll1-null embryo (del Barco Barrantes et al., 1999), suggesting that Dll1 might be involved in specifying both rostral and caudal characteristics. Expression of *Cer1* is usually observed as two stripes, finally localizing to the rostral half of nascent somites in the wild-type embryo (Fig. 2A). The stripe of *Cer1* expression is severely downregulated in both Dll1-null and Mesp2-null embryos, as well as in the Dll1/Mesp2 double-null embryo (Fig. 2B-D), suggesting that Dll1 and Mesp2 lie in the same cascade in regulating expression of rostral marker genes. Although *Dll1* expression is expanded in the absence of Mesp2, no *Cer1* induction is observed, suggesting that *Cer1* is not directly induced by Dll1 but by Mesp2.

We next observed the expression pattern of the caudal half marker gene, *Uncx4.1*. Normal stripes of *Uncx4.1* expression are completely lost in the Dll1-null embryo, indicating that *Dll1* lies upstream of *Uncx4.1* (Fig. 2E,F). In the Mesp2-null embryo, expression of both *Dll1* and *Uncx4.1* is strongly expanded, suggesting the involvement of Dll1 in the expansion of *Uncx4.1* expression (Fig. 2G). If only Dll1 specifies the caudal half property, as expected from our previous model, the lack of Mesp2 should not affect the loss of the caudal half property in the Dll1-null embryo. However, additional loss of *Mesp2* in the Dll1-null embryo results in the reappearance of *Uncx4.1* expression (Fig. 2H), indicating that *Uncx4.1* had been suppressed by Mesp2 in the Dll1-null embryo. *Mesp2* expression in the Dll1-null embryo is greatly reduced (Fig. 1H), but this trace amount of Mesp2 expression must be enough to suppress *Uncx4.1* expression. Therefore, even in the absence of Dll1, *Uncx4.1* is expressed in the somite region by loss of Mesp2 (Dll1/Mesp2 double-null embryo). However, the level of *Uncx4.1* expression is obviously higher in the Mesp2-null embryo than in the Dll1/Mesp2 double-null embryo,

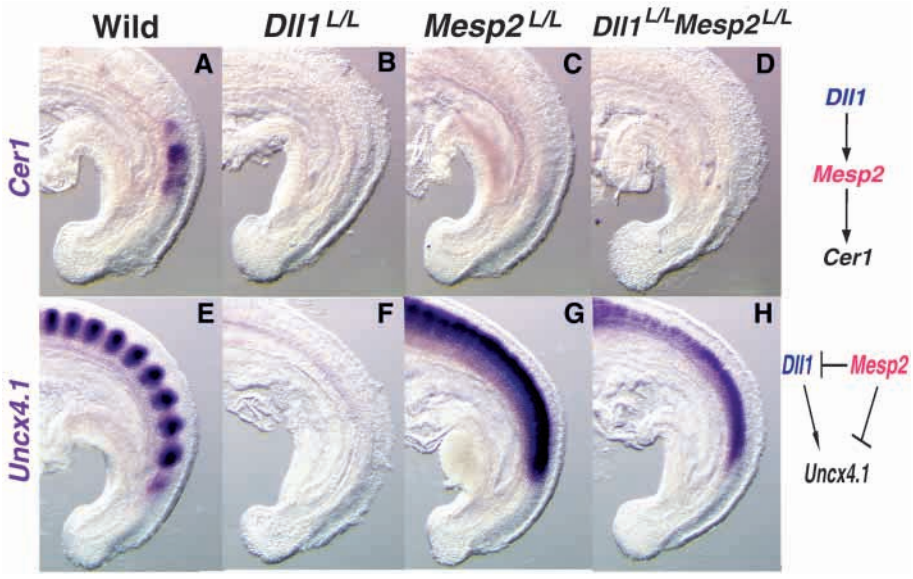


Fig. 2. Dll1 is required for normal expression of both rostral and caudal genes, and Mesp2 suppresses the caudal half property in both Dll1-dependent and Dll1-independent manners. Expression of *Cer1* is usually observed as two or three stripes, finally localizing to the rostral half of nascent somite in the wild-type embryo (A). *Cer1* expression is almost lost in both Dll1-null and Mesp2-null embryos (B,C), as well as the Dll1/Mesp2 double-null embryo (D). Normal stripes of *Uncx4.1* expression, localizing to the caudal half of each somite (E), are completely lost in the Dll1-null embryo (F). In Mesp2-null embryos, expression of both *Dll1* (Fig. 1) and *Uncx4.1* is strongly expanded (G). However, the additional loss of *Mesp2* in the Dll1-null embryo results in an expanded pattern of *Uncx4.1* expression (H). Genetic cascades are also shown.

showing that Dll1 can induce *Uncx4.1* in the absence of Mesp2. This indicates that *Uncx4.1* is induced by Dll1, and is also suppressed by Mesp2 independently of Dll1. We conclude, therefore, that Mesp2 suppresses the caudal half property in both Dll1-dependent and Dll1-independent manners. Thus, the Dll1-null phenotype is not a default state, and Mesp2 function is required for the manifestation of the Dll1-null phenotype.

Dll3 and Mesp2 are required for normal expression of each other

Dll3 is the other Notch ligand expressed in the PSM, and its expression finally localizes to the rostral half of each somite (Dunwoodie et al., 1997). The *Pudgy* mutant (*Dll3$^{pu/pu}$*, Dll3-null) embryo exhibits expression of both rostral and caudal half marker genes, but the patterns are spatially disorganized (Kusumi et al., 1998). Thus, we cannot readily conclude from the pudgy phenotype alone whether the Dll3-Notch signal results in activation or suppression of *Dll1*. To explore the roles of Dll3 in formation of the rostrocaudal polarity of somites, we first examined the mutual regulation of *Dll3* and *Mesp2*. *Pudgy* is a frame-shift mutation caused by a four-nucleotide deletion (Kusumi et al., 1998), allowing us to analyze expression of *Dll3* transcript in the *Dll3$^{pu/pu}$* embryo. Comparison between wild and *Dll3$^{pu/pu}$* embryos has revealed that the rostral stripes of *Dll3* expression are lost in the absence of functional Dll3 (Fig. 3A,B) (Kusumi et al., 1998), indicating that Dll3 is required for formation of the stripe pattern of its own expression. A relatively clear boundary in the expression level was observed between the PSM and somite region in the *Dll3$^{pu/pu}$* embryo. The level of *Mesp2* expression is significantly decreased in the *Dll3$^{pu/pu}$* embryo, suggesting that Dll3 upregulates expression of *Mesp2* (Fig. 3C,D). Finally, in the Mesp2-null embryo, instead of stripe formation, a weak diffuse *Dll3* expression is expanded rostrally (Fig. 3E,F). The above observations show that Dll3 induces expression of *Dll3* itself and *Mesp2*, while Mesp2 suppresses expression of *Dll3*. Thus, the regulatory interactions between *Dll3* and *Mesp2* appear similar to those of *Dll1* and *Mesp2*. However, the expansion of *Dll3* expression in the absence of Mesp2 is also observed in the Dll3/Mesp2 double-null embryo, indicating that it does not depend on Dll3 (Fig. 3G-J). This situation is different from that for *Dll1* and *Mesp2* (Fig. 1Q). Thus, the regulatory relationship between *Dll3* and *Mesp2* is similar to but different from that between *Dll1* and *Mesp2*. Taken together, both *Dll3* and *Mesp2* are necessary for their mutual normal expression. This indicates that stripe pattern of *Dll3*, as well as that of *Dll1*, is formed by involvement of Mesp2, and not simply by the molecular clock oscillating in the posterior PSM.

Mesp2 genetically lies downstream of *Dll3* regarding rostrocaudal polarity

Next, we analyzed genetic interaction between *Dll3* and *Mesp2* to elucidate their hierarchy during formation of the rostrocaudal polarity. (For the rostral genes, see supplemental Fig. S1 at http://dev.biologists.org/supplemental/.) The rostrocaudal patterning defects in the *Dll3$^{pu/pu}$* embryo (Kusumi et al., 1998) and in the Mesp2-null embryo (Takahashi et al., 2000) have been previously reported, but we compared four genotypes (wild-type, *Dll3$^{pu/pu}$*, Mesp2 null, Dll3/Mesp2 double null) among our littermates for the precise evaluation of the double-null embryos. In the wild-type embryo, expression of *Dll1* is localized in the caudal half of each somite, with strong expression in the caudal PSM (Fig. 4A). However, only weak, blurred and randomized expression, instead of normal definite stripes, is seen in the somite region of the *Dll3$^{pu/pu}$* embryo (Fig. 4B). In the Mesp2-null embryo, strong expression of *Dll1* is expanded rostrally (Fig. 4C). The Dll3/Mesp2 double-null embryo exhibited expansion of strong *Dll1* expression, indistinguishable from that in the Mesp2-null embryo (Fig. 4D). *Uncx4.1* expression is also localized in the caudal half of formed somites in the wild-type embryo (Fig. 4E). The *Dll3$^{pu/pu}$* embryo exhibits a blurred and disorganized (salt-and-pepper) pattern of *Uncx4.1* expression (Fig. 4F), while the Mesp2-null embryo exhibits strong expansion of

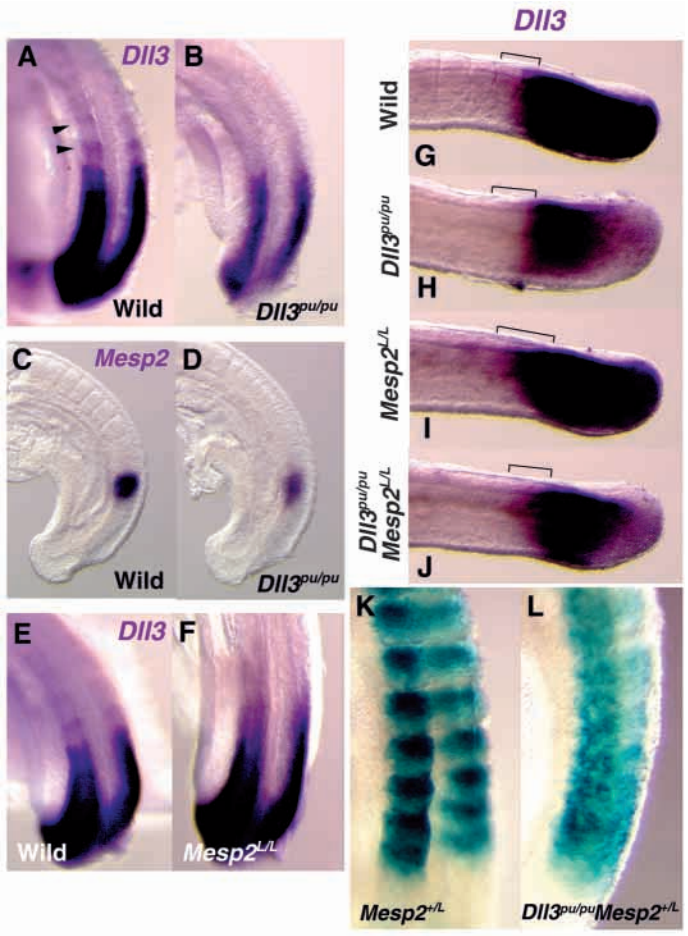


Fig. 3. Dll3 and Mesp2 are required for normal expression of each other. In the wild-type embryo at 9.5 dpc, expression of *Dll3* is finally localized to the rostral half of each somite (A). The *Dll3* stripe (arrowhead in A) is missing in the *Dll3$^{pu/pu}$* embryo (B). The level of *Mesp2* expression is significantly decreased in the *Dll3$^{pu/pu}$* embryo (C,D). In the Mesp2-null embryo, a weak diffuse *Dll3* expression is expanded rostrally (E,F). (G-J) Expansion of *Dll3* expression in the Mesp2-null embryo does not require Dll3. At 11.5 dpc, in the *Dll3$^{pu/pu}$* embryo, the *Dll3* stripe is missing and the expression is not expanded rostrally (G,H). Expansion of *Dll3* expression in the Mesp2-null embryo is not largely affected by the loss of Dll3 (I,J). (K,L) Dll3 is required for localization of *Mesp2* expression into the rostral half of somites. In the wild type, β-gal activity for *Mesp2-lacZ* is localized in the rostral half of somites (K). A randomized salt-and-pepper pattern is observed in the *Dll3$^{pu/pu}$* embryo (L).

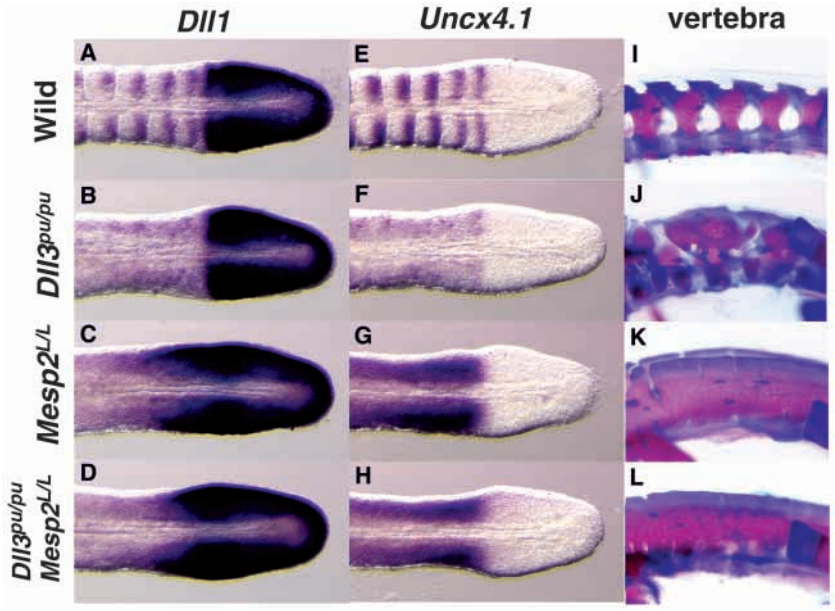


Fig. 4. Genetically, *Mesp2* lies downstream of *Dll3* regarding the rostrocaudal polarity. Expression of the caudal genes *Dll1* (A-D), *Uncx4.1* (E-H) and the morphology of the lumbar vertebrae (I-L) are examined in the Dll3/Mesp2 intercross. Genotypes are indicated on the left. The *Dll3*/*Mesp2* double-null embryo exhibits phenotypes indistinguishable from those of the Mesp2-null embryo. Details are stated in the text. For the rostral genes, see Fig. S1 at http://dev.biologists.org/supplemental/.

Uncx4.1 expression (Fig. 4G). As with *Dll1*, the Dll3/Mesp2 double-null embryo shows an *Uncx4.1* expression pattern indistinguishable from that in the Mesp2-null embryo (Fig. 4H). Finally, we examined the skeletal morphology of the lumbar vertebra. The pedicles and the laminae of the neural arches are arranged metamerically in the wild-type vertebral column (Fig. 4I). The *Dll3*$^{pu/pu}$ vertebrae show disorganized skeletal elements, partially fused to each other (Fig. 4J). The pedicles and the laminae are almost completely fused in the Mesp2-null fetus (Fig. 4K). The Dll3/Mesp2 double-null vertebrae exhibit almost completely fused neural arches (Fig. 4L). These observations indicate that *Mesp2* genetically lies downstream of *Dll3*, and that the salt-and-pepper pattern of *Uncx4.1* expression in the Dll3-null embryo requires the function of Mesp2. In other words, Mesp2 functions independent of Dll3 to suppress the caudal genes, *Dll1* and *Uncx4.1*, while Dll3 function is mediated by Mesp2. To know the function of Dll3 on Mesp2-mediated suppression on caudal genes, we further investigate their relationship. As Mesp2 is active in the *Dll3*$^{pu/pu}$ embryo with the salt-and-pepper pattern of *Dll1* and *Uncx4.1* expression, and localization of Mesp2 is crucial for rostrocaudal patterning, we examined the localization of *Mesp2-lacZ* expression in the *Dll3*$^{pu/pu}$ background by X-gal staining (Fig. 3K,L). Although expression of *Mesp2* mRNA at the rostral PSM simply seems moderately reduced and blurred (Fig. 3D), β-galactosidase activity in the somite region exhibited a salt-and-pepper pattern, instead of normal rostrally-localizing stripes (Fig. 3K,L). Thus, one major function of Dll3 is to localize expression of *Mesp2*.

Dll1-Notch signaling consists of both Psen1-dependent and Psen1-independent pathways

We have previously demonstrated that Mesp2-null and Psen1-null embryos exhibit contrastive rostrocaudal polarity of somites (Takahashi et al., 2000). To define whether Psen1 is involved in the Dll1-Notch or Dll3-Notch signaling pathway, we examined genetic interactions between *Psen1* and *Dll1* or *Dll3*. Examination of *Uncx4.1* expression in Dll1/Psen1 intercrosses proved that *Uncx4.1* expression is lost in both Dll1 and Psen1-null embryos, as well as in the Dll1/Psen1 double-null embryo (Fig. 5A-D). Therefore the induction of the caudal marker *Uncx4.1* is probably mediated by the Psen1-dependent Dll1-Notch signals. By contrast, the stripe expression of the rostral marker *Cer1* is only slightly decreased and expanded in the Psen1-null embryo, whereas it is almost lost in the Dll1-null embryo (Fig. 5E-G). The expanded *Cer1* expression in the Psen1-null embryo is lost by the additional loss of *Dll1* (the Dll1/Psen1 double-null embryo, Fig. 5H), implying that it is induced by the Psen1-independent Dll1-Notch signaling. The same result was obtained with the other rostral marker genes, *Epha4* and *Hoxd1* (data not shown). As Dll1 is required for the normal level of *Mesp2* expression that induces the expression of rostral genes, the requirement of Dll1 is likely to reflect the induction of *Mesp2*. Actually, expression of *Mesp2* is correlated with those of *Cer1* and *Epha4* (Fig. 5I-L). As *Mesp2* expression is moderately reduced in the Psen1-null embryo and is severely down-regulated in the Dll1/Psen1 double-null embryo, induction of *Mesp2* is likely to be mediated by both Psen1-dependent and Psen1-independent Notch signaling. These observations suggest that at least Psen1-independent Dll1-Notch signaling induces *Mesp2* and thereby rostral genes such as *Cer1*. However, both Dll1 and Dll3 contribute to the Psen1-dependent signals. Therefore, we analyzed the interaction of *Dll3* and *Psen1*.

Dll3-Notch signals are also both Psen1-dependent and Psen1-independent

The expression level of *Mesp2* was moderately decreased in the Dll3-null, Psen1-null and Dll3/Psen1 double-null embryos, and they were comparable among the three genotypes, suggesting that *Mesp2* expression is partly dependent on Psen1-dependent Dll3-Notch signaling (Fig. 5M-P). However, the remaining *Mesp2* expression observed in Dll3/Psen1 double-null embryo is dependent on neither Dll3 nor Psen1, confirming that this expression of *Mesp2* is induced via Psen1-independent Dll1-Notch signaling as already suggested (Fig. 5).

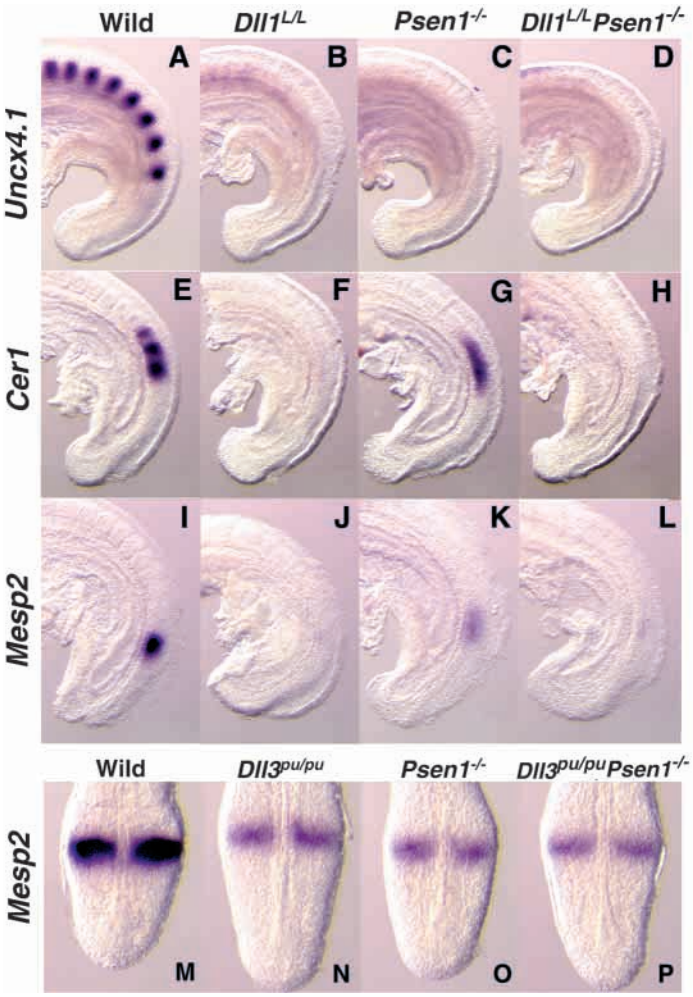


Fig. 5. Dll1-Notch signaling consists of both Psen1-dependent and Psen1-independent pathways. Normal *Uncx4.1* expression (A) is lost in both Dll1 (B) and Psen1-null (C) embryos, as well as in Dll1/Psen1 double-null embryo (D). The stripe expression of the rostral marker *Cer1* (E) is almost lost in the Dll1-null embryo (F), whereas it is expanded in the Psen1-null embryo (G). This expanded expression is lost by the additional loss of *Dll1* (the Dll1/Psen1 double-null embryo, H). Likewise, *Mesp2* expression (I) is almost lost in the Dll1-null embryo (J), moderately reduced in the Psen1-null embryo (K) and is almost lost in the Dll1/Psen1-double null embryo (L). (M-P) *Mesp2* expression is partly dependent on Psen1-dependent Dll3-Notch signaling. When compared with the wild type (M), expression levels of Mesp2 are decreased in the Dll3-null (N), Psen1-null (O) and Dll3/Psen1 double-null (P) embryos, and they are comparable among the three genotypes.

The expression patterns of caudal marker genes were correlated with the morphology of the vertebrae (Fig. 6). In the *Psen1*-null embryo ($Dll3^{+/+}Psen1^{-/-}$), stripes of *Dll1* and *Uncx4.1* expression were completely lost, and the pedicles of the neural arches were missing (Fig. 6C,H,M) (Takahashi et al., 2000). Although blurred *Dll1* expression was not detected, weak disorganized *Uncx4.1* expression was observed in the Dll3/Psen1 double-null embryo ($Dll3^{pu/pu}Psen1^{-/-}$, Fig. 6D,I). This level of *Uncx4.1* expression was lower than that in the $Dll3^{pu/pu}$, but distinguishable from that in the $Dll3^{+/+}Psen1^{-/-}$. This suggests that Dll3 can suppress expression of *Dll1* and *Uncx4.1* in the absence of Psen1, and Psen1 can mediate the Dll1-Notch signal to induce expression of *Dll1* and *Uncx4.1* in the absence of Dll3. These are further confirmed by the analyses of skeletal phenotypes. The vertebrae of $Dll3^{pu/pu}Psen1^{-/-}$ exhibited an intermediate morphology between $Dll3^{pu/pu}$ and $Dll3^{+/+}Psen1^{-/-}$ vertebrae. Whereas the $Dll3^{pu/pu}$ vertebrae had a considerable amount of disorganized skeletal elements in the position of the pedicles (Fig. 6L), the amount of disorganized skeletal elements was smaller in the vertebrae of $Dll3^{pu/pu}Psen1^{-/-}$ (Fig. 6N). Thus, the phenotype of $Dll3^{pu/pu}Psen1^{-/-}$ embryos differs from the phenotypes of both $Dll3^{pu/pu}$ and $Dll3^{+/+}Psen1^{-/-}$ embryos.

Dll3 and *Psen1* can counteract each other

Surprisingly, the loss of one copy of *Dll3* in the Psen1-null embryo restored the stripe pattern of gene expression. The $Dll3^{+/pu}Psen1^{-/-}$ embryo exhibited faint stripes of *Dll1* and *Uncx4.1* expression (Fig. 6E,J), and a small amount of skeletal elements at the position of the pedicles, although not regularly arranged (Fig. 6O). This indicates that Psen1-mediated Dll1-Notch signals and Dll3-Notch signals counteract each other in regulating *Dll1* and *Uncx4.1* expression, and establishing rostrocaudal polarity. In other words, the stripe pattern of gene expression is formed on a balance of two counteracting signals. Taken together, Dll3 and Psen1 can function independently, and have at least in some cases, opposite functions. This is also demonstrated in the morphology of the proximal ribs (see supplemental Fig. S2 at http://dev.biologists.org/supplemental/).

DISCUSSION

Dll1, Dll3 and Psen1 differentially regulate the rostrocaudal polarity of somites

Our results on involvement of Dll1, Dll3, Mesp2 and Psen1 in establishment of the rostrocaudal polarity are summarized in Fig. 7A. The present findings clarify the ligands for Notch signaling pathways in our previous model. *Dll1* is activated by the Psen1-dependent Dll1-Notch signaling pathway and suppressed by the Psen1-independent Dll3-Notch pathway. However, this suppressive Dll3 pathway is not sufficiently active in the absence of Mesp2. Mesp2 plays a major role in suppression of the caudal genes, including *Uncx4.1*, more directly than Dll1 or Dll3. In our previous model, (1) rostral localization of *Mesp2* expression is given a priori and (2) Dll1 exclusively specifies caudal half properties. However, the present scheme shows that both Dll1 and Dll3 influence the expression of *Mesp2*. Thus, these genes constitute a complex network, and interactions among these genes result in the simultaneous localization of *Dll1*, *Dll3* and *Mesp2*. In addition, Dll1-Notch signal is required for both rostral and caudal properties, as it induces *Dll1* itself and *Mesp2*. In contrast to Dll1, Dll3 upregulates *Mesp2* and suppresses *Dll1* and *Uncx4.1*, resulting in the suppression of caudal half properties. This is the first report specifying the functional differences of Dll1 and Dll3 in somite patterning. It should be noted, however, that the scheme in Fig. 7A does not immediately represent signaling cascades within single cells, but instead represents results from complex intercellular interactions among mesodermal cells in the rostral PSM.

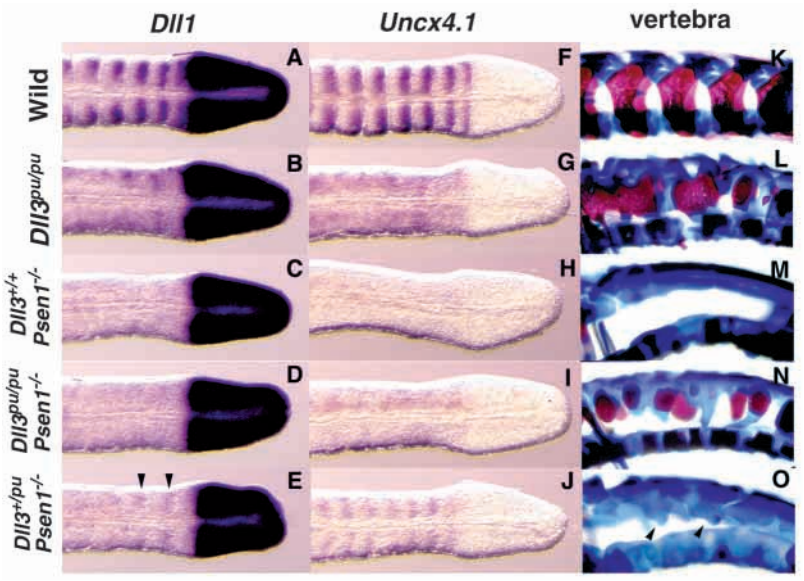
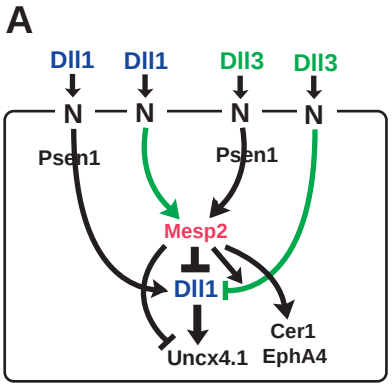


Fig. 6. Dll3 and Psen1 can act independently of each other in regulation of the caudal genes. The stripe pattern of *Dll1* and *Uncx4.1* is correlated with the skeletal morphology of the vertebrae (A,F,K). In the *Dll3*$^{pu/pu}$ embryo, the blurred and randomized expression of *Dll1* and *Uncx4.1* results in disorganized skeletal elements (B,G,L). In the Psen1-null embryo (*Dll3*$^{+/+}$*Psen1*$^{-/-}$), stripes of *Dll1* and *Uncx4.1* expression, and the pedicles were completely lost (C,H,M). Weak disorganized expression of *Uncx4.1* was observed in the Dll3/Psen1 double-null embryo (*Dll3*$^{pu/pu}$*Psen1*$^{-/-}$; D,I). The vertebrae of *Dll3*$^{pu/pu}$*Psen1*$^{-/-}$ exhibited an intermediate morphology between Dll3-null and Psen1-null vertebrae (N). Surprisingly, the *Dll3*$^{+/pu}$*Psen1*$^{-/-}$ embryo exhibited faint stripes of *Dll1* (E, arrowheads) and *Uncx4.1* (J), and a small amount of skeletal elements (O, arrowheads).



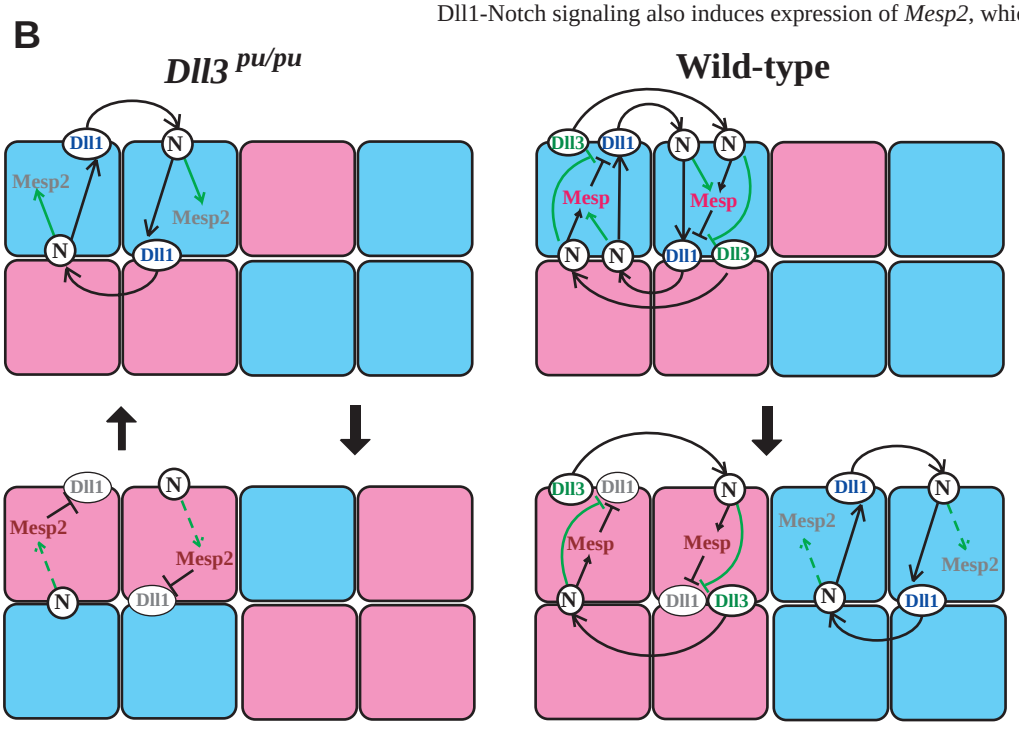


Fig. 7. (A) Summary of putative signaling cascades in the anterior PSM. The Psen1-independent pathways are shown with green arrows. Dll1-Notch signaling results in induction of both *Dll1* itself and *Mesp2*. The positive feedback of *Dll1* is mediated by the Psen1-dependent signal. Induction of *Mesp2* is mediated via Psen1-independent Dll1-Notch signaling and Psen1-dependent Dll3-Notch signaling. In contrast to Dll1, Dll3 has roles in upregulation of *Mesp2* and suppression of *Dll1* and *Uncx4.1*. (B) Integration of stripe pattern by Dll3 function. For simplification, anterior PSM cells of four-cell width are illustrated. Pink cells represent the dominance of the Mesp2 function, and blue cells the dominance of the Dll1 function. Genes and arrows are shown only between two representative cells for simplicity. The green arrows show the Psen1-independent pathways and broken lines show inactive states. Even in the absence of Dll3, Dll1-Notch signaling and Mesp2 are still active (left). Reciprocal Dll1-Notch signaling between two neighboring cells results in induction of *Dll1* in both cells. Meanwhile, reciprocal Dll1-Notch signaling also induces expression of *Mesp2*, which suppresses expression of *Dll1* cell-autonomously in both cells. When *Dll1* is downregulated, Mesp2 is also reduced by the lack of the juxtacrine Dll1 signal. Thus, the positive and negative feedback loops of *Dll1* and *Mesp2* produce uneven spatial patterns of Dll1 and Mesp2, but fail to form integrated stripe patterns in the absence of Dll3. Although the precise mechanism is unknown, participation of Dll3 results in synchronization of Dll1-dominant and Mesp2-dominant cells by suppressing *Dll1* expression in cooperation with Mesp2 (right). After segregation, Dll3 and Mesp2 continue to suppress *Dll1* and *Uncx4.1* expression in the rostral half, while Dll1 induces expression of *Dll1* itself and *Uncx4.1* via Psen1-dependent pathway in the caudal half. In the caudal half, induction of *Mesp2* expression via Psen1-independent pathway is inactive.

Stripe formation in the anterior PSM and oscillation in the posterior PSM

Expression of some genes considered to reflect the molecular clock, such as chick *hairy1*, oscillates as a 'traveling wave' in the posterior PSM, stabilizes at the anterior PSM and finally forms a half-a-somite stripe retained in somites (Palmeirim et al., 1997). Therefore, the rostrocaudal patterning, i.e. formation of half-a-somite stripe pattern of gene expression, has been regarded as a result of stabilization of oscillating expression in the posterior PSM. However, our analysis of the mutual regulation of *Dll1*, *Dll3* and *Mesp2* has demonstrated that none of the half-a-somite stripe patterns of *Dll1*, *Dll3* and *Mesp2-lacZ* are formed in the absence of Mesp2 function (Figs 1, 3). In particular, expression of *Mesp2-lacZ* is strongly expanded in the Mesp2-null embryo, implying that expression of *Mesp2* does not simply conform to the stripe prepattern formed by the molecular clock. This is in contrast to the stripes of *Uncx4.1-lacZ* in the absence of Uncx4.1 function (Mansouri et al., 2000), where expression of *Uncx4.1-lacZ* faithfully reflects the stripe prepattern formed in advance. At present, there is no evidence of the half-a-somite stripe prepattern upstream of *Mesp2*.

There is another example that the oscillation in the posterior PSM seems to be separated from the stripe formation. Holley et al. (Holley et al., 2002) have reported the interesting observation that in zebrafish embryos injected with her1-MO, a normal stripe of *deltaC* expression is formed in the anterior PSM, in the absence of oscillation of *deltaC* or *her1* in the posterior PSM. In this case, the *deltaC* stripe at the anterior PSM is not a result of simple stabilization of oscillating expression in the posterior PSM, but is likely to be formed by another mechanism. This stripe formation also appears to be mediated by Notch signaling, because the additional loss of DeltaD function disrupts stripe formation. In addition, injection of her1/her7 double-MO completely abolishes stripe formation (Oates and Ho, 2002). Holley et al. suggested that Notch signaling acts in oscillation of cyclic gene expression in the posterior PSM as well as in stripe formation (refinement of the stripe) at the anterior PSM. We propose that the narrowing stripe is formed at the anterior PSM, by the positive and negative feedback loops among *Dll1*, *Dll3* and *Mesp2*. These feedback loops may constitute a kind of cellular oscillator in the anterior PSM, which is distinct from the oscillator in the posterior PSM (Fig. 7B). This process may be normally linked with the oscillation process in the posterior PSM.

Interpretation of the salt-and-pepper pattern and possible functions of Dll3

The remarkably randomized and chaotic nature of vertebrae in the pudgy mouse has long been a mystery for geneticists. The salt-and-pepper pattern of gene expression in the Dll3-null mouse embryo is similar to that in zebrafish mutants *aei*, *des* and *bea*. Jiang et al. (Jiang et al., 2000) attributed this salt-and-pepper pattern to a desynchronized oscillator activity in individual cells, and concluded that Notch signaling is not essential for the oscillator activity itself, as the salt-and-pepper pattern is regarded as a result of a complete lack of Notch function in zebrafish mutants. However, we have demonstrated by genetic analysis that both Dll1-Notch signaling via Psen1 (Fig. 6) and Mesp2 (Fig. 4) are functioning in the Dll3-null embryo (Fig. 7B). We propose a model for rostrocaudal patterning, where the positive and negative feedback loops of *Dll1* and *Mesp2* and their integration by Dll3 are essential (Fig. 7B). Even in the absence of Dll3, *Dll1* and *Mesp2* are still expressed at considerable levels, and interactions among adjacent cells can result in two different states. The Dll1-Notch signal activates expression of *Dll1* in neighboring cells, thus causing upregulation of *Dll1* in a group of cells. Subsequently, the reciprocal Dll1-Notch signal also induces *Mesp2* expression, which suppresses *Dll1* expression so that *Dll1* is downregulated in the cell population. When *Dll1* is downregulated, *Mesp2* levels are also reduced by the lack of the juxtacrine Dll1 signal. Thus, the cells can 'oscillate' between the two states in the absence of Dll3. With some impact of initial stochastic activation, these interactions may produce and maintain uneven salt-and-pepper patterns of gene expression. In the wild-type embryo, involvement of Dll3 leads to synchronization of Dll1-dominant and Mesp2-dominant cells, and thus integration of the stripe pattern. As Mesp2 functions to activate rostral properties and suppresses caudal properties, the Mesp2-dominant domain is referred to as the presumptive rostral domain. The current model is a further development of our previous model (Takahashi et al., 2000). In our previous paper we showed that the stripe of *Dll1* expression is not a remainder of strong expression in the posterior PSM, but is newly induced via Psen1-dependent Notch signaling. That is, all the cells spanning the future one somite region undergo suppression by Mesp2, and the *Dll1* stripe is formed after or simultaneously with this suppression. We now interpret this process to be a result of the integration of cellular oscillation at the individual cellular level.

What then is the synchronizing function of Dll3 at the cellular level? The salt-and-pepper pattern of *Dll1* and *Uncx4.1* expression in the $Dll3^{pu/pu}$ embryo has somewhat confused the issue of whether the Dll3-Notch signal activates or suppresses *Dll1* expression. As the level of blurred and mislocalized *Dll1* expression in the $Dll3^{pu/pu}$ embryo is lower than that of definite *Dll1* stripes in the wild-type embryo, one might consider that Dll3 function is required for activation of *Dll1*. However, strong expansion of *Dll1* expression is evident in the Dll3/Mesp2 double-null embryo, as well as in the Mesp2-null embryo, indicating that Dll3 is not necessary for the auto-activation of *Dll1* via a positive feedback loop. Although the precise mechanism leading to the synchronization is yet to be defined, the likely function of Dll3 is to suppress Dll1-Notch signaling, probably in cooperation with Mesp2. This function seems feasible when considered in relation to their normal expression patterns, as the expression of *Dll3* and *Mesp2* finally localizes to the rostral half. Actually, the restoration of the stripe pattern of *Dll1* and *Uncx4.1* in the $Dll3^{+/pu}Psen1^{-/-}$ embryo implies that Dll3-Notch signaling can counteract Psen1-dependent Dll1-Notch signaling. This phenomenon also suggests that the stripe pattern is formed by a balance of two opposing signals. Probably, the requirement of Psen1 for the activation of *Dll1* is not absolute, and in the Psen1-null embryo, a severely reduced, weak ability for *Dll1* activation is overcome by suppression by Dll3-Notch signaling. Thus, reduction of the amount of the Dll3-Notch signal may restore the balance of the counteracting signals.

In the posterior PSM, Dll1 and Dll3 have essential roles in formation of traveling waves of cyclic genes such as lunatic fringe and *Hes1* (del Barco Barrantes et al., 1999; Jouve et al.,

2000; Dunwoodie et al., 2002). Therefore, we cannot exclude the possibility that Dll1 and Dll3 influence the rostrocaudal patterning through their effects on the molecular clock in the posterior PSM. Analysis of the possible linkage between stripe formation at the anterior PSM and the oscillation process in the posterior PSM is of special importance for understanding the roles of Notch signaling in somitogenesis.

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