Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse

Iván del Barco Barrantes*†, Andrew J. Elia*†, Kurt Wünsch‡, Martin Hrabe De Angelis‡, Tak W. Mak*†, Janet Rossant§¶, Ronald A. Conlon¥, Achim Gossler# and José Luis de la Pompa*†°

Background: The process of somitogenesis can be divided into three major events: the prepatterning of the mesoderm; the formation of boundaries between the prospective somites; and the cellular differentiation of the somites. Expression and functional studies have demonstrated the involvement of the murine Notch pathway in somitogenesis, although its precise role in this process is not yet well understood. We examined the effect of mutations in the Notch pathway elements *Delta like 1* (*Dll1*), *Notch1* and *RBPJκ* on genes expressed in the presomitic mesoderm (PSM) and have defined the spatial relationships of Notch pathway gene expression in this region.

Results: We have shown that expression of Notch pathway genes in the PSM overlaps in the region where the boundary between the posterior and anterior halves of two consecutive somites will form. The *Dll1*, *Notch1* and *RBPJκ* mutations disrupt the expression of *Lunatic fringe (L-fng)*, *Jagged1*, *Mesp1*, *Mesp2* and *Hes5* in the PSM. Furthermore, expression of *EphA4*, *mCer 1* and *uncx4.1*, markers for the anterior–posterior subdivisions of the somites, is down-regulated to different extents in Notch pathway mutants, indicating a global alteration of pattern in the PSM.

Conclusions: We propose a model for the mechanism of somite border formation in which the activity of Notch in the PSM is restricted by *L-fng* to a boundary-forming territory in the posterior half of the prospective somite. In this region, Notch function activates a set of genes that are involved in boundary formation and anterior–posterior somite identity.

Addresses: *Amgen Institute, 620 University Avenue, Toronto, Ontario M5G 2C1, Canada. †Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario M5G 2C1, Canada. ‡GSF, Institute for Mammalian Genetics, Ingolstaedter Landstrasse 1, D-85764 Oberschleissheim, Germany. §Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. ¶Departments of Molecular and Medical Genetics, and Obstetrics and Gynecology, University of Toronto, Ontario, Canada. ¥Department of Genetics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4955, USA. #The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA.

Present address: °EMBL, Mouse Biology Programme, via Ramarini 32, I-00016 Monterotondo Scalo, Rome, Italy.

Correspondence: José Luis de la Pompa
E-mail: delapompa@embl-monterotondo.it

Received: 21 December 1998
Revised: 4 March 1999
Accepted: 25 March 1999

Published: 22 April 1999

Current Biology 1999, 9:470–480
http://biomednet.com/elecref/0960982200900470

Background

Somites, paired blocks of mesodermal cells that are arranged bilaterally on either side of the neural tube, represent one of the earliest signs of metamerism in the mammalian embryo [1]. They form in a strict cranio-caudal order by the successive segmentation of the paraxial mesoderm. Caudal to the most recently formed somite, the paraxial mesoderm appears morphologically unsegmented. This tissue is known as the presomitic mesoderm (PSM) and is contiguous with the tissue at the caudal end of the embryonic axis. Somites are subdivided into anterior and posterior halves that differ in their adhesive properties and gene expression (reviewed in [2,3]). This alternation of anterior and posterior properties patterns spinal ganglia and nerves, and also constitutes a mechanism to maintain the borders between segments [4,5]. Embryonic manipulations demonstrated that the anterior–posterior (a–p) polarity of the somites is established in the PSM prior to the formation of distinct somites [6]. In addition, gene expression studies indicate that the apparently homogenous PSM is subdivided into domains of distinct gene expression, which in the anterior PSM correlate with the future a–p somite halves and/or with the boundaries of nascent somites (reviewed in [2,3]).

There is increasing experimental evidence that cell-to-cell communication mediated by the evolutionary-conserved Notch signalling pathway is of functional significance for somite development. Homologues of Notch pathway genes have been identified in the mouse, including *Delta like 1* (*Dll1*) [7] and *Delta like 3* (*Dll3*) [8], which are homologues of the ligand gene *Delta* [9]; *Jagged 1* and *2* (*Jag1* and *Jag2*) [10–12], which are homologues of the ligand gene *Serrate* [13]; *Notch1–4* [14–17], which are homologues

of the receptor gene *Notch* [18]; *Lunatic fringe* (*L-fng*), *manic fringe* and *radical fringe*, which are homologues of *fringe* (*fng)* [19,20]; and *RBPJκ* (recombination signal sequence binding protein for *Jκ* genes), which is a homologue of *Suppressor of Hairy* (*Su(H))* [21,22]. For reviews on Notch signalling, see [23–25]. The somite defects of mice with targeted or spontaneous mutations in Notch pathway elements have been characterized to varying degrees. Mice carrying mutations for *Notch 1* [26,27], *RBPJκ* [28], *Dll1* [5] or *Dll3* [29] have somites with irregular size and shape; in *Dll1* and *Dll3* mutants, the a–p polarity and epithelialization of somites is affected. Given that *L-fng* is expressed at the forming somite borders [19,20] and that somitogenesis is disrupted in *L- fng* mutant mice [30,31], it has been suggested that Notch signalling might regulate somite boundary formation and wing margin development in *Drosophila* through similar molecular mechanisms. In *Drosophila*, interactions between dorsal and ventral cells organize the wing around a discrete dorsal–ventral (d–v) boundary, the wing margin. Fringe controls the formation of the wing margin by regulating the signalling activity of Serrate and Delta at this border [32,33].

Somitogenesis is a continuous process that generates new borders at at relatively constant species-specific rate. On the basis of experiments with amphibian embryos, a 'clock and wavefront' model has been proposed to account for the properties of somite formation. According to this model, groups of cells oscillate synchronously between two states driven by a cellular 'clock' while they are in the PSM. A wavefront of maturation sweeps back along the embryo in an anterior→posterior direction and cells at the anterior end of the PSM that cycle together form a somite once the wave front has passed [34]. This model is also supported by evidence obtained from studies showing the rhythmic expression in the PSM of *c-hairy 1*, the chicken homologue of *Drosophila hairy* [35], and chicken and mouse *L-fng* [36,37]. These findings suggest a link between Notch signalling and the molecular clock driving somite formation, although the nature of this connection is unknown.

Here, we have studied the consequences of perturbed Notch signalling in the PSM of *Dll1*, *Notch1* and *RBPJκ* mutants. Mutations in *Dll1* and *RBPJκ*, but not in *Notch1*, disrupt patterning of the PSM and a–p somite polarity is lost, as indicated by the severe down-regulation of the anterior somite markers *EphA4* and *mCer 1*, and of the posterior marker *uncx4.1*. We show that the stripes of *Jag1*, *L-fng* and of the PSM basic helix–loop–helix (bHLH) genes *Mesp2* and *Hes5* expression overlap with those of *Dll1* and *Notch1* in the PSM. Furthermore, *Dll1*, *Notch1* and *RBPJκ* are required for the expression of *L-fng*, suggesting that *L-fng* is a target of Notch activity in the PSM. Likewise, mutations in *Dll1*, *Notch1* and *RBPJκ* affect the striped expression in the PSM of *Hes5*, *Jag1*, *Mesp1* and *Mesp2*, indicating that expression of these genes is regulated by Notch activity. We suggest that Notch signalling in the anterior PSM is restricted by L-Fng to the interface between the prospective posterior somite half and the anterior half of the next developing somite, and we propose a model of how boundary formation might occur.

Results

Disruption of gene expression and patterning in the PSM of Notch pathway mutants

Mesoderm segmentation in the mouse embryo begins around embryonic day 8 (E8), with the formation of somites. Analysis of mouse mutants lacking *Notch1* [27], *Dll1* [5] or *RBPJκ* (I.dB.B. and J.L.dlP., unpublished observations) suggests that these genes are not essential for cellular differentiation in the paraxial mesoderm because somite derivatives develop. Rather, Notch signalling appears to be required for proper somite formation and early patterning.

To find targets of Notch activity in the PSM, we studied the expression of genes that are potentially involved in either Notch signalling or the transition from PSM to segmented somites. We analyzed the expression of these genes in the mutants *Dll1*, *Notch1* and *RBPJκ*, to discriminate functional differences between this ligand, receptor and effector that might be due to redundancy or different target specificity.

In *Drosophila*, the *Enhancer of split* genes (encoding bHLH proteins) are targets of Notch signalling [38–40]. In mammals, the *Hes* genes (*Hes1–5*) are related to both the *Hairy* and *Enhancer of split* genes (reviewed in [41]). The *Hes* genes are regulated by Notch signalling *in vitro* [42] and *in vivo*, as indicated by the effect of *Notch1* and *RBPJκ* mutations on the expression of *Hes5* [43]. To determine whether the absence of *Dll1* affects the transcription of the same targets, we analyzed *Hes* expression in *Dll1* mutants by whole mount *in situ* hybridization. Among the different *Hes* genes expressed at E8.5–9.0 (*Hes1*, *Hes3* and *Hes5*), only *Hes5* was severely down-regulated in the PSM, and was less affected in the central nervous system (CNS) ($n = 5$; compare Figure 1a,c with Figure 1b,d). This phenotype is similar to that found in *RBPJκ* or *Notch1* mutants [43], indicating that the bHLH gene *Hes5* is a common target for Dll1, Notch1 and RBPJκ in both the PSM and the CNS.

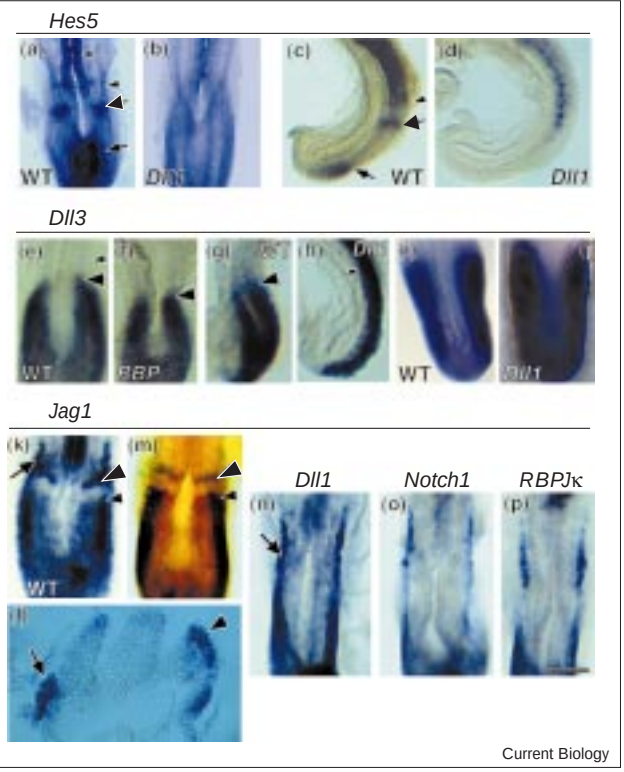
In *Drosophila*, Notch signalling is known to regulate the expression of its ligands [44]. We have shown previously that *Dll1* is severely down-regulated in the posterior somite halves of *RBPJκ* and *Notch1* mutant embryos, although expression in the PSM appears unaffected [43]. Similarly, the expression of *Dll3*, a second mouse *Delta* gene expressed in the PSM ([8]; Figure 1e,g) and CNS [8] appeared normal in E8.5 *RBPJκ* ($n = 7$; Figure 1f), E8.5 *Notch1* (data not shown) and E9 *Dll1* mutants ($n = 8$;

Figure 1h). In contrast, at E10.5, *Dll3* transcript levels in *Dll1* mutants appeared high compared with wild type (n = 5; compare Figure 1i with Figure 1j). In addition, *Dll3* was strongly up-regulated in the CNS of *Dll1* mutants (n = 8; Figure 1h).

The Serrate-type ligand Jag1 is expressed during somitogenesis [10,12,45,30]. At E8.5, *Jag1* is expressed in the PSM, in one stripe in the most recently formed somite, in the PSM and in a long band flanking the paraxial mesoderm (Figure 1k). Sections of whole mount stained embryos reveal that this band corresponds to the intermediate mesoderm (Figure 1l). Double label *in situ* hybridization shows that *Jag1* expression overlaps with *Dll1* in the PSM and in the posterior half of the youngest somite (Figure 1m). *Jag1* expression in the PSM and in the newly formed somite is severely reduced in the Notch pathway mutants *Dll1* (n = 6; Figure 1n), *Notch1* (n = 6; Figure 1o) and *RBPJκ* (n = 7; Figure 1p), suggesting that its expression depends on Notch activity.

L-fng is the only murine *fringe* homologue expressed in the paraxial mesoderm [19,20]. Forsberg *et al.* [36] have made a comprehensive analysis of the waves of mouse *L-fng* expression in the PSM, and showed that a new somite boundary forms just after the end of a wave. To determine whether the dynamic transcription of *L-fng* in the PSM was affected in Notch pathway mutants, we compared *L-fng* expression in groups of wild-type and mutant embryos. Figure 2a–f shows representative phases of the temporal and spatial cycle of *L-fng* expression in wild-type embryos. The widest domain of *L-fng* expression spans two stripes in the anterior PSM, with the most anterior stripe narrower than the posterior one, and a large area of mesoderm adjacent to the primitive streak (Figure 2a) [36]. After reaching their maximal cranial extension, transcripts gradually disappear posteriorly (Figure 2b), leaving only the two anterior stripes (Figure 2c), which progressively refine (Figure 2d). Subsequently, expression in the posterior region is reinitiated (Figure 2e), and the more anterior stripe of *L-fng* expression disappears (Figure 2f). Posterior to it, a new somite boundary will form [36]. In *RBPJκ* (n = 17; Figure 2g–l) and *Dll1* (n = 14; Figure 2n–r,t,u) mutants, we observed a very severe down-regulation of *L- fng* in the PSM. In a few cases, a single, very faint stripe of expression was detected (Figure 2g), but never the characteristic range of dynamic *L-fng* expression domains. In contrast, *L-fng* expression was only slightly reduced and had less defined borders in *Notch1* mutants (n = 9; Figure 2v–z). These data suggest that *L-fng* is a target of Notch signalling in the PSM. *L-fng* expression in the CNS appears to be unaffected, implying that in this tissue, *L-fng* may be regulated independently of Notch activity, and that Notch is differently regulated during lateral inhibition in the CNS and inductive signalling in the PSM.

Figure 1



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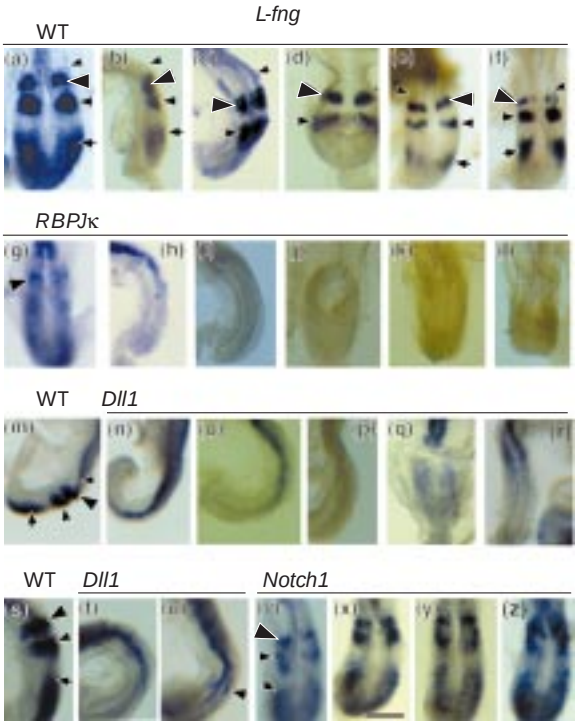
Abnormal *Hes5*, *Dll3* and *Jag1* expression in Notch pathway mutants (whole mount *in situ* hybridization, details of posterior regions) **(a)** E8.5 wild-type (WT) embryo. *Hes5* is expressed in the PSM (thick arrow), primitive streak (thin arrow) and neural tube (small arrow). **(b)** E8.5 *Dll1* mutant showing a severe down-regulation of *Hes5* in the PSM and neural tube. **(c)** An E9 wild-type embryo maintains *Hes5* expression in the PSM (thick arrow), primitive streak (thin arrow) and neural tube (the small arrowhead points to the most posterior osmite boundary), whereas **(d)** a *Dll1* mutant shows no signal in the PSM and streak and reduced expression in the neural tube. **(e)** E8.5 wild-type embryo. *Dll3* is strongly expressed in the PSM, particularly at the anterior end (large arrowhead). **(f)** E8.5 *RBPJκ* mutant with normal *Dll3* expression (arrowhead). **(g)** At E9 in the wild-type embryo, *Dll3* expression in the PSM is maintained (arrowhead) and weak patchy expression is observed in the neural tube. **(h)** In contrast, E9 *Dll1* mutant embryos show apparently normal *Dll3* expression in the PSM, and a strong up-regulation in the neural tube (arrow). **(i)** Expression of *Dll3* in the tailbud region of an E10.5 wild-type embryo. **(j)** Increased *Dll3* expression in the tailbud of an E10.5 *Dll1* embryo. **(k)** In wild-type E8.5 embryos, *Jag1* is expressed in the newly formed somite (large arrowhead), PSM (small arrowhead), intermediate mesoderm (arrow), and neural tube. **(l)** A transverse section showing *Jag1* expression in the dermomyotome (arrowhead) and intermediate mesoderm (arrow) of a wild-type embryo. **(m)** Double label *in situ* hybridization shows that *Dll1* expression (red) overlaps with *Jag1* (blue) in the PSM (small arrowhead), and in the posterior half of the newly formed somite (large arrowhead). *Jag1* expression in the PSM and youngest somite is severely reduced in **(n)** *Dll1*, **(o)** *Notch1* and **(p)** *RBPJκ* mutant embryos, although the signal in the intermediate mesoderm appears less affected (arrow). The small arrowhead in (a,c,e) points to the most posterior somite boundary. The scale bar represents 70 μm in (a–h,n–p), 90 μm in (i–m) and 50 μm in (l). (a,b,e,f,i–k,m,n–p) are dorsal views; (c,d,g,h) are lateral views; (l) is a transverse section.

Figure 2



Disruption of *L-fng* expression in Notch pathway mutants. Selected phases of the *L-fng* wave of expression in the PSM. (a) When the wave is well-advanced, the widest domain of *L-fng* spans two stripes in the PSM, and a large area of the primitive streak. Gradually, signal in the primitive streak is (b) down-regulated and (c) finally disappears, and (d) the two anterior stripes refine. Progressively, signal in the primitive streak reinitiates (e,f), and (f) the first stripe of *L-fng* expression down-regulates, finally disappearing once a boundary forms posterior to it, at the end of the wave. In (a–c,e,f) the small arrowhead points to the most posterior somite boundary, and in (a–f) the large and mid-size arrowheads point to the first two stripes of expression in the PSM. The arrow in (a,b,e,f) points to the signal in the streak. In (c,d) the signal in the streak is downregulated. In (d) the posterior somite boundary is not indicated. (g–l) Examples of *L-fng* expression in *RBPJκ* mutant embryos. Only the embryo in (g) shows a faint stripe of expression (arrowhead). (m) Wild-type embryo, showing extended *L-fng* expression. (n–r,t) *Dll1* mutants show no sign of expression or (u) very reduced signal in the PSM (arrowhead). (s) A wild-type embryo with three areas of *L-fng* expression in the PSM (arrowheads) and streak (arrow). (v–z) *Notch1* mutants show a reduction of *L-fng* signal in (v) the PSM (arrowheads) and in the streak (arrow). The scale bar represents 70 μm. (a,d–g,j–l,q,r,v–z) are dorsal views; (b,c,h,i,m–p,s–u) are lateral views.

The bHLH transcription factor Mesp2 is required for normal mesoderm segmentation in the mouse [46]. *Mesp2* is transcribed in the anterior region of the PSM (Figure 3a–d). Double label *in situ* hybridization has shown that *Mesp2* expression overlaps with the anterior region of *Dll1* expression [46]. *Mesp2* is severely down-regulated in the PSM of *Dll1* ($n = 8$; Figure 3e) and *RBPJκ* ($n = 7$; Figure 3f) mutants, but is much less affected in *Notch1* mutants ($n = 5$; Figure 3g,h). Similar results were obtained with the other family member, *Mesp1* [47] (data not shown), indicating that *Mesp1* and *Mesp2* expression depends on Notch signalling activity.

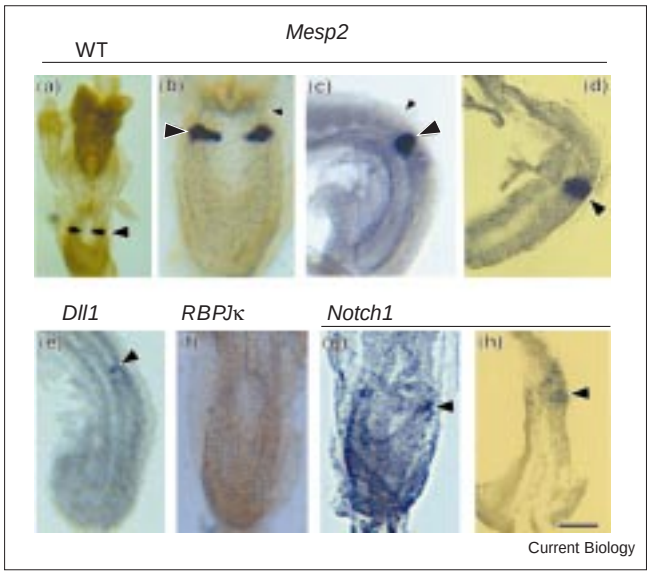
Dll1 and *RBPJκ* are essential for anterior–posterior somite polarity

To address whether somite polarity is similarly affected in *Dll1*, *RBPJκ* and *Notch1* mutant embryos, we analyzed the expression of *uncx4.1*, which encodes a paired homeodomain protein related to *Caenorrhabditis elegans* unc4 [48,49]. *Uncx4.1* is expressed in the posterior half of the somites (Figure 4a–c), as sagittal sections demonstrate (Figure 4d). *Uncx4.1* expression is not detected in E8.5 *Dll1* mutants ($n = 8$; Figure 4e), reinforcing our earlier interpretation that the identity of posterior somite halves is lost [5]. Similarly, *Uncx4.1* expression is absent in *RBPJκ* mutants ($n = 9$; Figure 4f). In contrast, *uncx4.1* transcripts are detected in the posterior somite halves of *Notch1* mutant embryos ($n = 5$; Figure 4g,h). Consistent with this observation, expression of *Pax1* in both the anterior and posterior sclerotomal halves (Figure 4i,j) is relatively normal in *Notch1* mutants ($n = 4$; Figure 4k,l).

To address whether the perturbation of Notch activity causes a global disruption of the segmental pattern in the PSM, we examined the expression of *EphA4* and *mCer 1* in mutant embryos. The receptor tyrosine kinase (RTK) EphA4 (Sek1) [50] belongs to a large family of RTKs that have been implicated in axonal pathfinding through a short-range contact-mediated guidance mechanism (reviewed in [51]). *EphA4* is dynamically expressed in the PSM. Initially, transcripts are abundant in one stripe of approximately the size of one somite, and in the primitive streak (Figure 5a). Expression in the somitic region becomes progressively refined and restricted to the anterior half of the prospective somite, and a broader expression domain appears posteriorly (Figure 5b) [50]. Eventually, expression in the most anterior stripe is down-regulated, and the posterior stripe refines (Figure 5c). Once the epithelial somite is formed, the anterior stripe disappears [50]. Double label *in situ* hybridization shows that the first stripe of *EphA4* expression, which marks the anterior half of the prospective somite [50], is rostral to the first stripe of *L-fng* expression (Figure 5d). Sagittal sections demonstrate the expression of *L-fng* in the anterior half of the newly formed somite and in the PSM (Figure 5e). *EphA4* expression is down-regulated in the PSM of *Dll1* ($n = 10$; Figure 5f), *RBPJκ* ($n = 12$; Figure 5g) and *Notch1* ($n = 11$; Figure 5h) mutants, although the effect in the latter appears less severe, as indicated by sagittal sections (Figure 5i).

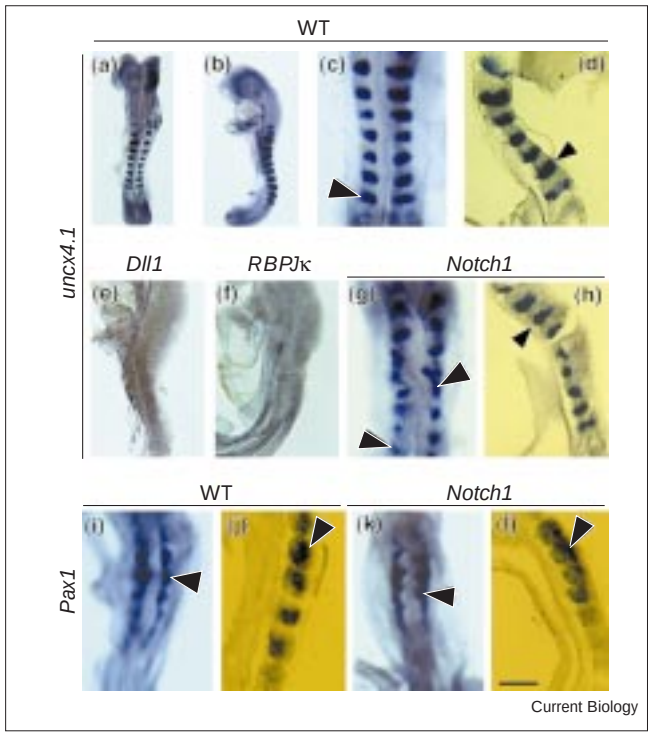
The mCer 1 protein [52,53] is the murine homologue of *Xenopus* Cerberus (XCer), a member of the transforming growth factor-β (TGFβ) and bone morphogenetic protein (BMP) superfamily with anterior patterning properties, as

Figure 3



Down-regulation of *Mesp2* expression in Notch pathway mutants. Whole mount *in situ* hybridizations. At (a,b) E8.5 and (c) E9, *Mesp2* is found in one stripe in the anterior border of the PSM (large arrowheads), as illustrated by (d) a sagittal section through an E8.5 embryo (large arrowhead). The small arrowheads point to the most posterior somite boundary. *Mesp2* signal is (e) drastically decreased in *Dll1* (large arrowhead), (f) undetectable in *RBPJκ* mutants, and (g) reduced in *Notch1* mutants (large arrowhead), as (h) a sagittal section shows (arrowhead). The scale bar represents 200 μm in (a), 70 μm in (b–g) and 50 μm in (h).

Figure 4



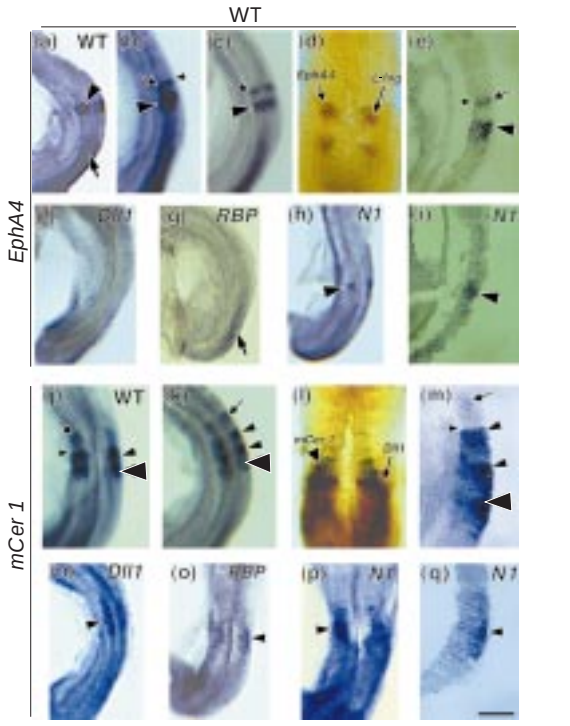
Loss of *uncx4.1* expression in *Dll1* and *RBPJκ* mutants. Whole mount *in situ* hybridizations. (a–c) The *uncx4.1* gene is expressed at E8.5–9 in the posterior somite half (arrowhead in (c)). (d) Parasagittal section through an E8.5 wild-type *in situ* hybridized embryo showing *uncx4.1* expression in the posterior somite half (arrowhead). The expression of *uncx4.1* is severely down-regulated in (e) *Dll1* and (f) *RBPJκ* mutants. (g) *Notch1* embryos show expression of *uncx4.1* in the posterior somite halves (left arrowhead), although in some somites, the *uncx4.1* expression domain appears wider (right arrowhead). (h) Sagittal sections through *Notch1* mutant embryos reveal the posterior somite restriction of *uncx4.1* (arrowhead). In comparison, *Pax1* mRNA is detected in the anterior and posterior somite halves in both (i,j) wild-type (arrowheads) and (k,l) *Notch1* (arrowheads) mutant embryos. In (l), the arrowhead points to the poorly defined somites of the *Notch1* mutant. The scale bar represents 200 μm in (a,b) and 70 μm in (c–l). (a,c,g,i,k) are dorsal views; (b,e,f) are lateral views; (d,h,j,l) are sagittal sections.

revealed by misexpression studies [54]. In the E8.5–8.75 mouse, *mCer 1* is expressed in the PSM and nascent somites (Figure 5j,k) [52]. In the PSM, *mCer 1* expression occupies a two-somite-wide domain and marks the anterior region of the two newest somites (Figure 5k) [52]. Double staining with *mCer 1* and *Dll1* probes shows that the anterior domain of *mCer 1* is anterior to the limit of *Dll1* signal in the PSM (Figure 5l). Histological sections show that the expression of *mCer 1* is down-regulated in mature somites, anteriorly restricted in the newly formed somite, and occupies two stripes within the PSM (Figure 5m). Mutants of the Notch pathway show reduced and poorly defined *mCer 1* expression. This is particularly the case with *Dll1* ($n = 7$; Figure 5n) and *RBPJκ* ($n = 9$; Figure 5o). Notch1 mutants show a weak *mCer 1* expression in the PSM ($n = 6$; Figure 5p,q). These results demonstrate that mutations in *Dll1* and *RBPJκ* disrupt the compartmentalization of somites, whereas Notch1 function appears to be non-essential for the establishment of a–p somite polarity.

Overlapping expression domains of Notch signalling elements in the PSM suggests regulatory interactions

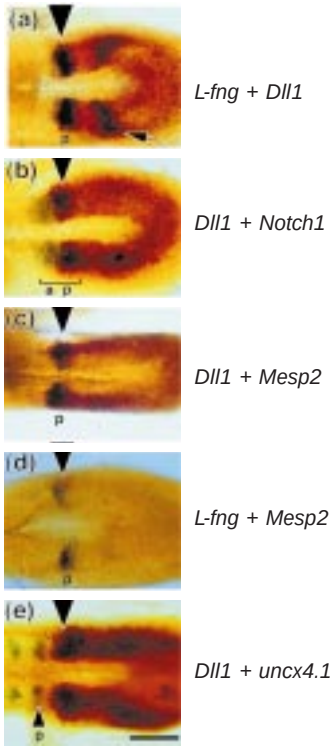
Our results show that a number of genes expressed in distinct PSM regions and nascent somites depend on Notch signalling for their expression. In order to define precisely the expression domains of putative Notch target genes with respect to the ligand *Dll1* and the receptor *Notch1*, and to determine whether they could be regulated by Notch signalling, we carried out double label *in situ* hybridization experiments in wild-type embryos. We did not assay the expression of *RBPJκ*, as it is ubiquitously expressed throughout the PSM and somites [28]. At the end of a wave of expression, the first stripe of *L-fng* in the PSM overlaps with the strongest domain of *Dll1* expression in the anterior border of the PSM ($n = 7$; Figure 6a), demarcating the prospective posterior somite half ('p'). This observation is consistent with previous data [20]. *Dll1* and *Notch1* expression overlap with each other in the anterior PSM ($n = 7$; Figure 6b), and the strongest domain of *Notch1* expression is

Figure 5



Disruption of *EphA4* and *mCer 1* expression in Notch pathway mutants. (a) At E8.5–9, *EphA4* is expressed in a somite-size stripe in the PSM (arrowhead) and in the primitive streak (arrow). (b) Once the somite is generated, signal is restricted to the anterior half (arrow). A strong stripe of expression appears in the border of the PSM (large arrowhead). (c) Transcription in the newly formed somite is progressively down-regulated (arrow) and the posterior PSM stripe becomes more refined (arrowhead). (d) Double label *in situ* hybridization shows that *EphA4* expression (blue) is rostral to the first stripe of *L-fng* (red) in the PSM. (e) Sagittal section showing reduced *EphA4* expression in the anterior half of the newly formed somite (arrow) and a strong stripe in the border of the PSM (large arrowhead). *EphA4* signal is severely reduced in the PSM of E9 (f) *Dll1* and (g) *RBPJκ* mutants, and reduced in (h) *Notch1* (arrowhead). (i) Sagittal section showing reduced *EphA4* expression in the PSM of a *Notch1* embryo. The arrowhead points to a reduced *EphA4* expression in the PSM. (j) At E8.5, *mCer 1* is expressed in one stripe in the newly formed somite (large arrowhead), in the anterior half of the preceding somite (arrow), and in one stripe in the PSM (thick arrowhead). (k) E9 wild-type embryo, with expression in the anterior PSM (thick arrowhead), in the anterior halves of two newly formed somites (large arrowheads), and clear anteriorly restricted expression in an older somite (arrow). (l) Double label *in situ* showing *mCer 1* expression (blue, thick arrowhead) anterior to the border of *Dll1* signal (red, arrow) in the PSM. (m) Sagittal section showing strong *EphA4* signal in the PSM (thick arrowhead), anteriorly restricted expression in the two preceding somites (large arrowheads), and strongly reduced signal in the anterior half of an older somite (arrow). The small arrowheads in (b,e,j,m) point to the most posterior somite boundaries. *mCer 1* signal is reduced and poorly defined in the PSM and somitic region of (n) *Dll1* embryos (large arrowhead), and severely down-regulated in (o) *RBPJκ* mutants (large arrowhead). (p,q) *Notch1* mutants also show reduced and poorly defined *mCer 1* expression in the PSM region (arrowheads). The scale bar represents 70 μm in (a–d,f–h,j–l,n–p) and 50 μm in (e,i,m,q). (d,l,n–p) are dorsal views; (e,i,m,q) sagittal sections; (a–c, f–h,j,k) are lateral views.

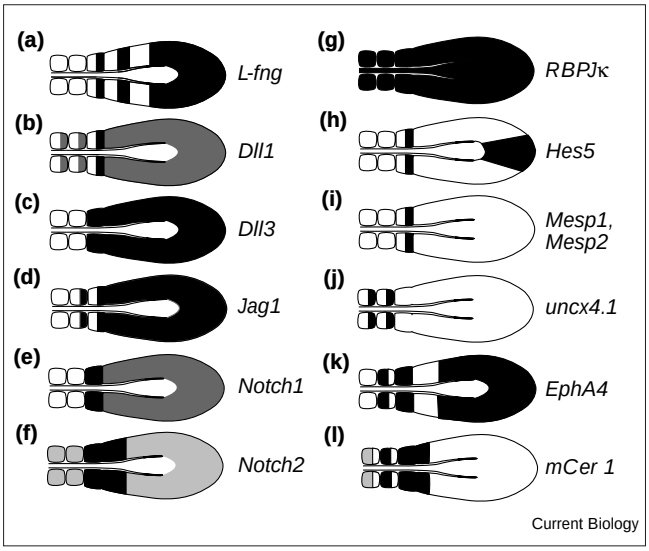
Figure 6



Overlapping expression domains of Notch pathway elements in the PSM and somites. Double label whole mount *in situ* hybridization on E8.5 wild-type embryos. Dorsal views of the PSM and tail region; anterior is to the left. (a) Double label with *L-fng* (blue) and *Dll1* (red) probes. The arrowhead points to the overlap (brown) at the end of a wave of *L-fng* expression in the PSM, between the anterior *L-fng* stripe and the anterior border of *Dll1* expression. 'p' denotes the posterior prospective somite half. Note the second *L-fng* stripe in the PSM (arrow). (b) Double label with *Dll1* (red) and *Notch1* (blue) probes. The arrowhead points to the overlap between *Dll1* and *Notch1* in the anterior PSM. The bracket indicates a somite-wide domain of expression demarcated anteriorly (a) by *Notch1* and posteriorly (p) by the overlap between *Notch1* and *Dll1*. (c) Double label with *Dll1* (red) and *Mesp2* (blue) probes. The arrowhead indicates the overlap between *Dll1* and *Mesp2* in the PSM, which is consistent with the co-expression of (d) *L-fng* and *Mesp2* (arrowhead) in this same region, the prospective posterior somite half ('p'). (e) Double label with *Dll1* (red) and *uncx4.1* (blue). The large arrowhead points to the border of *Dll1* expression in the PSM and the small arrowhead to the overlap between *Dll1* and *uncx4.1* in the posterior half of the somites. The scale bar represents 70 μm.

anterior to *Dll1*, overlapping with *Dll3* in the most anterior region of the PSM ('a'; data not shown). Thus, *L-fng*, *Dll1*, *Dll3*, *Notch1* (and *RBPJκ*) are all expressed in the prospective posterior somite half. Together, the strongest *Dll1* and *Notch1* expression domains in the PSM are the approximate size of a somite (Figure 6b). Double stainings with *Dll1* and putative target genes in the PSM show that their expression overlaps in the PSM region where the posterior somite

Figure 7



Summary of gene expression in the PSM. Posterior mesodermal tissues are illustrated in dorsal view with anterior to the left. The intensity of shading is meant to represent differences in gene expression levels for a gene, but is not meant to imply differences in expression levels between genes. (a) Snapshot of the widest domain of *L-fng* dynamic expression in the PSM: a first stripe of expression corresponding to the posterior half of the prospective somite, a second broader stripe midway through the PSM, and a third region in the primitive streak. (b) *Dll1* is expressed in the posterior half of the prospective and mature somites, PSM and primitive streak. (c) *Dll3* is expressed homogeneously throughout the PSM, surpassing the anterior limit of *Dll1* expression in the prospective somite. (d) *Jag1* is expressed in the posterior half of the epithelial and prospective somite and throughout the PSM. (e) *Notch1* is expressed at high levels in the prospective somite and more weakly throughout the PSM and primitive streak. (f) *Notch2* is expressed similarly to *Notch1*, but is also expressed in recently formed somites. (g) *RBPJκ* is ubiquitously expressed. (h) *Hes5* is expressed in one stripe in the posterior half of the prospective somite and in the streak. (i) *Mesp1* and *Mesp2* are expressed in the posterior half of the prospective somite. (j) *Uncx4.1* is expressed in the posterior halves of mature somites. (k) *EphA4* is expressed in the anterior half of the epithelial somite, in a somite-wide domain, and in the primitive streak. (l) *mCer 1* is expressed in the anterior halves of the two most recently formed somites and in two stripes in the PSM. The expression patterns, determined by *in situ* hybridization in this study, were compared with those reported in the following references: *L-fng* [20,60], *Dll1* [7], *Dll3* [8], *Jag1* [30,45], *Notch1* [59], *Notch2* [46], *RBPJκ* [28], *Hes5* [43], *Mesp1* [47], *Mesp2* [46], *uncx4.1* [49], *EphA4* [50] and *mCer 1* [52].

boundary forms. Thus, the stripe of *Mesp2* expression coincides with the anterior border of *Dll1* expression in the PSM, delineating the prospective posterior somite half ($n = 5$; Figure 6c). Consistently, *Mesp2* overlaps with the anterior stripe of *L-fng* expression in the PSM ($n = 4$; Figure 6d). *Dll1* and the anterior stripe of *Hes5* expression also coincide in the same PSM region (data not shown). Furthermore, *Dll1* and *uncx4.1* transcripts overlap in the posterior region of the newly formed somite ($n = 5$; Figure 6e). Figure 7 shows a summary of the wild-type expression patterns in the PSM and somites of the genes analyzed. Table 1 summarizes the effects on the expression of PSM and/or somite markers caused by mutations in Notch pathway genes.

Table 1

Expression analysis of regionalized PSM and/or somite markers in Notch pathway mutants.

	Genotype					
	L-fng	*Dll1*	*Dll3*	*Notch1*	*RBPJκ*	*Mesp2*
Marker						
PSM + streak — dynamic						
L-fng	–	SDR	DB*	DR	SDR	ND
Anterior PSM						
Mesp1	ND	SDR	ND	DR	SDR	+
Mesp2	ND	SDR	ND	DR	SDR	–
PSM + streak						
Dll1	DB†,‡	–	+	+	+	SDR§
Dll3	DB†,‡	UR	–	+	+	ND
Jag1	+	DR	ND	DR	DR	ND
Notch1	DB†,‡	+¥	ND	–	+¥	DR§
Notch2	DB†,‡	+¥	ND	ND	+¥	DR§
PSM + posterior streak						
Hes5	SDR‡	SDR	ND	DR#	SDR#	ND
Posterior half epithelial somite						
Jag1	DB†	SDR	ND	SDR	SDR	ND
Anterior somite half						
EphA4	ND	SDR	ND	DR	SDR	ND
mCer 1	ND	DR	ND	DR	DR	ND
Posterior somite half						
Uncx4.1	DB†,‡	SDR	ND	DR	SDR	ND
Dll1	DB†,‡	–	DB*	DR#	SDR#	DB§

Abbreviations: +, normal; DB, diffuse boundaries; DR, down-regulated; ND, not determined; SDR, severely down-regulated; UR, up-regulated. References to the original papers are indicated. The data for *Dll1*, *Notch1* and *RBPJκ* mutants are presented in this paper, except when indicated: *[29], †[30], ‡[31], §[46], ¥I.dB.B. and J.L.dlP., data not shown, and #[43].

Discussion

Loss of anterior–posterior somite identity in Notch pathway mutants

The perturbation of Notch signalling activity causes a general patterning disruption in the PSM, and the subdivision of somites into anterior and posterior compartments is severely perturbed, as indicated by the abnormal expression of markers for both the anterior and posterior somite halves. *EphA4* is dynamically expressed in two stripes in the PSM. The first one delimits the anterior half of the prospective somite and is down-regulated after the epithelial somite is formed. The second stripe spans a somite-sized domain [50]. Expression of dominant-negative forms of *EphA4* in zebrafish embryos blocks somite segmentation and affects normal *Delta* expression in the anterior

PSM [55]. The generation of a mouse mutant for *EphA4*, however, has not revealed a role for this gene in somitogenesis [56], perhaps due to the expression of other Eph receptor genes in this tissue [57]. *EphA4* is severely down-regulated in Notch pathway mutants. Double label *in situ* hybridization shows that *EphA4* expression abuts anteriorly the first stripe of *L-fng*, which defines the posterior somite half [20,36]. Similar to *EphA4*, *mCer 1* is expressed in two stripes in the PSM and in the anterior halves of the two newest somites [52]. It has been suggested that mCer 1 is involved in providing positional cues to the spinal cord or neural crest. In Notch pathway mutants, *mCer 1* expression is reduced, and the limits of its expression domain are less defined.

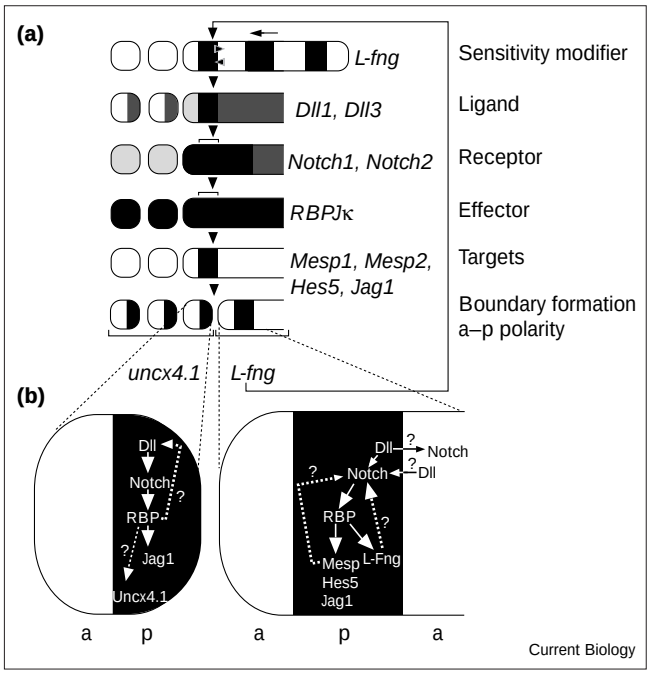
Expression of *Dll3* is up-regulated in the PSM and CNS of *Dll1* embryos, suggesting that *Dll1* negatively regulates *Dll3* expression. In addition, *Dll3* mutants do not express *Dll1* in the posterior somite halves [29], which implies that *Dll3* has a role in the circuitry establishing a–p somite identity in the PSM. *Dll1* is absent in the posterior somite halves of *RBPJκ* mutants [43], suggesting that in the somites, *Dll1* expression and RBPJκ activity might be linked by a positive feedback loop (see Figure 8). We cannot, however, rule out the possibility that the loss of *Dll1* expression could be an indirect consequence of the perturbed somite polarity in *RBPJκ* mutants. Likewise, *Jag1* is markedly down-regulated in the PSM and in the posterior half of the newly formed somite in *Dll1*, *Notch1* and *RBPJκ* mutants, indicating that Notch activity regulates *Jag1* expression in the PSM. The transcription factor *uncx4.1* is expressed in the posterior half of the newly formed somite, but not in the PSM, being one of the earliest markers of posterior somite identity [49]. Expression of *uncx4.1* is drastically down-regulated in *Dll1* and *RBPJκ*, and to a lesser extent in *Notch1* mutants. Whether *uncx4.1* is a target of Notch involved in the establishment of posterior somite identity, or is expressed as a consequence of compartmentalization, is not clear at present.

In our studies, the *RBPJκ* and *Dll1* mutations show more severe effects on gene expression than does the *Notch1* mutation, indicating functional redundancy at the level of the receptor. The fact that boundaries are formed in even the most severe case (*RBPJκ*) suggests that the generation of distinct a–p somite compartments is not essential for intersomitic boundary formation. Thus, Notch signalling appears to function in positioning the boundaries, not in their formation per se.

A model for Notch signalling activity in the PSM

We think that our data are consistent with the model of Notch activity during somite boundary formation presented in Figure 8. This model is based on our results and data from several laboratories on *L-fng* expression [19,20], its cyclic pattern in the PSM [36,37], and its *in vivo* function

Figure 8



A model for the generation of somite boundaries and a–p somite polarity by the Notch signalling pathway. This model does not attempt to elucidate the *L-fng* cyclic pattern in the PSM, but only to explain the effects of Notch pathway mutations on genes like *L-fng*, expressed in the anterior PSM. **(a)** The anterior, more developmentally advanced somites are shown to the left, the morphologically unsegmented PSM is on the right. At the end of a wave that progresses caudo-rostrally (arrow), *L-fng* is expressed in three stripes in the PSM and primitive streak (black). The first *L-fng* stripe coincides with the posterior half of the prospective somite. The shaded arrowheads indicate the interface between the posterior half of the prospective somite and the anterior half of the next somite. *L-fng* expression overlaps with the most anterior domain of *Dll1* in the PSM (black). *Dll3* mRNA reaches the anterior border of the PSM (light grey) and overlaps posteriorly with *Dll1* (black and grey). *Notch1* and *Notch2* expression overlaps with the ligands in the same territory (bracket), and extends rostrally and caudally in the PSM (see Figure 7). The boundary will form in this territory, perhaps because of the effect of L-Fng in the specific combination of ligand(s) and receptor(s) expressed here. RBPJκ is ubiquituously expressed (black) and Notch signalling may activate the genes *Mesp1*, *Mesp2*, *Hes5* and *Jag1* in the posterior region of the prospective somite. In this region, boundary formation occurs. Subsequently, *uncx4.1* (black) is transcribed in the posterior half of the newly formed somite, and its expression is maintained like that of *Dll1* throughout somite differentiation. The cycle of *L-fng* expression continues. **(b)** Putative elements of the Notch pathway in the PSM (right) and in the somites (left). Right: within the PSM domain defined by the first stripe of *L-fng* expression, Notch (Notch1 or Notch2) might receive the signal from the ligands Dll1 and Dll3 (perhaps also from across the prospective p–a boundary). The signal from Notch is transduced via RBPJκ and activates putative target genes, including *L-fng*. Dll (Dll1 or Dll3) might also signal to the anterior half of the next somite, activating the receptor Notch (Notch1 or Notch2). Left: in the posterior half of the epithelial somite, *Jag1* is expressed as a consequence of Notch signalling activity. This might also be the case for *uncx4.1* (dashed arrow). The large white arrows indicate transduction of signal (Dll to Notch to RBPJκ), or effects in gene expression (RBP on *Hes5* or *Mesp*). The thicker dashed arrows indicate positive feedback loops. a, anterior somite half; p, posterior somite half.

in the mouse [30,31]. The model takes into account the clock and wavefront model of somitogenesis [34] and the role of Fringe during wing margin development in *Drosophila* [32]. The model does not attempt to explain the cyclic pattern of *L-fng* expression, but only to integrate the observed phenotypes and effects on gene expression in Notch pathway mutants.

At the end of a wave of expression, *L-fng* is found in three stripes within the PSM, and a somite boundary forms just posterior to the anterior-most (first) *L-fng* stripe (Figure 8a) [36]. At this point of the wave, the first stripe of *L-fng* expression overlaps with the anterior limit of *Dll1* expression in the PSM ([20] and Figure 6a). *Dll3* transcripts are widely expressed in the PSM, and their expression domain overlaps with and extends rostrally to *Dll1* [8] and *L-fng*. *Notch1* and *Notch2* overlap with the anterior *L-fng* stripe. We suggest that boundary-promoting signalling occurs in the PSM territory where the first *L-fng* stripe overlaps with *Dll3*, *Dll1*, *Notch1* and *Notch2*. In this region, the boundary between the posterior half of the prospective somite and the anterior half of the next somite could be defined by the confrontation of *L-fng*-positive and *L-fng*-negative cells. Once the Notch receptor (Notch1 and/or Notch2) is activated in the posterior half of the prospective somite, the signal is transduced to the ubiquitously expressed RBPJκ, which in turn activates the potential bHLH target genes *Mesp1*, *Mesp2* and *Hes5*. Ultimately, changes in cellular properties, such as polarity and adhesion, might trigger or promote the formation of a boundary and generate a new somite. The cycle then starts again (Figure 8a).

We have found that *L-fng* is severely down-regulated in *Dll1*, *Notch1* and *RBPJκ* mutants, and *Dll3* mutants show less defined *L-fng* expression in the PSM [29]. On the other hand, the lack of *L-fng* leads to poorly defined anterior and posterior expression boundaries of *Dll1*, *Dll3*, *Notch1* and *Notch2*, consistent with a role for *L-fng* in positioning or spatially restricting Notch activity in the PSM [30,31]. Furthermore, *Hes5* expression is down-regulated in *L-fng* mutants [31], consistent with the idea that *L-fng* is required for high Notch activity. If, as in the *Drosophila* wing margin [32], *L-fng* acts upstream of Notch during somitogenesis, the expression of *L-fng* and *Notch* in the PSM might be linked by a positive feedback loop (Figure 8b). Alternatively, Notch signalling might be required solely to initiate the expression of *L-fng* in the PSM. Both *Dll1* and *RBPJκ* mutants show severely reduced expression of the bHLH genes *Mesp2* and *Mesp1* (data not shown) in the anterior PSM. In *Notch1* mutants, expression of these two genes is less affected, which is very likely to be because of functional compensation by *Notch2*. Gene targeting studies have revealed that *Mesp2* mutant mice show delayed somite segmentation, severe skeletal malformations, and caudal truncations [46]. Given that *Notch1* and *Notch2* expression was reduced in both the PSM and somites of *Mesp2* mutants, *Mesp2* was suggested to control *Notch* expression [46]. Our results, however, suggest that both *Mesp1* and *Mesp2* are targets of the Notch pathway. Notch lying upstream of both genes would be consistent with the normal *Mesp1* expression in *Mesp2* mutants [46], and the down-regulation of both *Mesp1* and *Mesp2* that we observe in Notch pathway mutants. The effect of the *Mesp2* mutation on the expression of *Notch1* and *Notch2* suggests also that the expression of these genes might be regulated by a positive feedback loop (Figure 8b).

The model draws from what is known about the role of Fringe in the regulation of Notch activity during wing margin development in *Drosophila* [32]. Differences between the developmental systems of mice and *Drosophila*, however, are apparent. During somite boundary formation, the effects on gene expression that we observe suggest that Notch signalling appears to occur in a large region corresponding to the posterior half of a forming somite, but not exclusively in cells that are adjacent to the boundary, as in the wing disc. Thus, Notch signalling in the PSM might establish a 'posterior domain' in the prospective somite, similar to the role of Notch during wing vein formation in *Drosophila* [58]. A role for Fringe in wing vein specification has not, however, been shown thus far.

Of the Notch pathway genes examined, *Dll1* [7], *Dll3* [8], *Notch1* [59], *Notch2* [16] and *RBPJκ* [28] are expressed in wide domains in the paraxial mesoderm that remain 'static' with respect to the somite-forming territory. In contrast, both in chicken [37] and in mouse [30], *L-fng* expression oscillates in the PSM in synchrony with the production of somites. Similarly, a study in the chick comparing *c-hairy 1* and *c-Delta1* expression in the PSM, has shown no evidence for a dynamic sequence of *c-Delta 1* mRNA distribution during somitogenesis [60]. We have found that *L-fng* expression in the PSM (like expression of *Hes5*, *Mesp1* and *Mesp2*) depends on Notch activity, suggesting that Notch signalling is required for at least some aspects of the 'read-out' of the molecular clock. The connection of Notch signalling with the 'clock' regulating somitogenesis, however, remains unclear.

Given that the anterior stripe of *L-fng* expression spans the posterior half of the prospective somite, why does the intersomitic boundary form at the p–a somite confrontation and not at the preceding a–p confrontation? This might depend on the effect of L-Fng on Notch activity and on the specific spatial combination of ligands and receptors that might allow the activation of the Notch pathway in this region, but not in the adjacent one. In addition, ligands localized in the anterior half of the next forming somite might activate/inhibit receptors expressed in the posterior half of the prospective somite (Figure 8b). A combination of genetic analysis and a precise immunohistochemical

definition of the distribution of the Notch pathway proteins will be required to resolve these issues.

Materials and methods

Genotyping

Dll1, *Notch1* and *RBPJκ* mutant embryos were obtained by mating females and males heterozygous for *Dll1* [5], *Notch1* [27] and *RBPJκ* [28] targeted mutations, respectively. Embryos were genotyped by PCR analysis of the yolk sacs. Primers and conditions were as described previously [5,27,28].

Whole mount in situ *hybridization*

Embryos were isolated in ice-cold phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde, and processed for whole mount *in situ* hybridization according to described procedures [61] with the modification that RNase treatment was omitted, except for the *Hes5* probe. Colour development was carried out using 0.45 µl of 125 mM nitroblue tetrazolium (NBT) solution and 3.5 µl of 115 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution per ml of NTMT (100 mM NaCl; 100 mM TrisHCl pH 9.5; 50 mM $MgCl_2$; 0.1% Tween-20) with 2 mM levamisole.

Double label *in situ* hybridization experiments were performed using a modification of previous protocols [8,20,46]. Riboprobes identifying the more strongly expressed gene product were labelled with fluorescein–UTP. Anti-fluorescein–alkaline phosphatase (AP) coupled antibody (Boehringer Mannheim) was used at 1:400. Colour development was carried out in the dark, overnight, at room temperature (RT) with SIGMA FAST. Twice the amount recommended by the manufacturer was used: 2.0 mg/ml of Fast Red TR; 0.8 mg/ml of Naphthol AS-MX; 0.15 mg/ml levamisol in 0.1 M Tris buffer. Embryos were subsequently washed at RT with PBT, fixed 20 min in 4% paraformaldehyde at RT, and washed twice with PBT at RT. The first antibody was inactivated by heating at 65°C for 30 min, embryos were blocked with 10% sheep serum for 30 min at 4°C, and incubated overnight at 4°C with the anti-DIG–AP coupled antibody at 1:500. After washing overnight in TBST, 2 mM levamisole, embryos were washed three times for 10 min in NTMT, 2 mM levamisole. Colour development was performed using 9 µl of NBT and 7 µl of BCIP per ml of NTMT with 2 mM levamisole. The following probes were used in this study: *Dll1* [7], *Dll3* [8], *Jag1* [30], *Hes5* [43], *L-fng* [19], *Mesp1* and *Mesp2* [46], *Uncx4.1* [49], *EphA4* [50] and *mCer 1* [52].

Histology

Hematoxylin and eosin staining was carried out in embryos fixed overnight in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned at 6 µm. After whole mount *in situ* hybridization, embryos were postfixed overnight in 4% paraformaldehyde, processed and sectioned at 20 µm.

Acknowledgements

We would like to thank S. Egan and L. Timmerman for critical reading and suggestions about the manuscript, J. Potter and M. Ng for technical assistance, T. Gridley for the *Jag1* probe, and D. Henrique, T. Vogt and Y. Saga for advice with the double label *in situs*. This work was supported by grants from the Medical Research Council of Canada to T.W.M., and of the US National Science Foundation to A.G. (IBN 9506156) and R.A.C. (IBN 9603627).

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