DEVELOPMENT AND DISEASE

Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene \textit{Dll3} are associated with disruption of the segmentation clock within the presomitic mesoderm

Sally L. Dunwoodie\textsuperscript{1,2,*}, Melanie Clements\textsuperscript{1}, Duncan B. Sparrow\textsuperscript{2}, Xin Sa\textsuperscript{3}, Ronald A. Conlon\textsuperscript{3} and Rosa S. P. Beddington\textsuperscript{1}

\textsuperscript{1}Division of Mammalian Development, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
\textsuperscript{2}Developmental Biology Unit, The Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst, NSW 2010, Australia
\textsuperscript{3}Department of Genetics, Case Western Reserve University, and University Hospitals Cleveland, 10900 Euclid Avenue, Cleveland, OH 44106-4955, USA

*Author for correspondence (e-mail: s.dunwoodie@victorchang.unsw.edu.au)

Accepted 14 December 2001

This article is dedicated to Rosa Beddington (March 23, 1956 to May 18, 2001) an extraordinary embryologist and a great friend

SUMMARY

A loss-of-function mutation in the mouse delta-like3 (\textit{Dll3}) gene has been generated following gene targeting, and results in severe axial skeletal defects. These defects, which consist of highly disorganised vertebrae and costal defects, are similar to those associated with the \textit{Dll3}-dependent pudgy mutant in mouse and with spondylocostal dysplasia (MIM 277300) in humans. This study demonstrates that \textit{Dll3}\textit{neo} and \textit{Dll3}\textit{pu} are functionally equivalent alleles with respect to the skeletal dysplasia, and we suggest that the three human \textit{DLL3} mutations associated with spondylocostal dysplasia are also functionally equivalent to the \textit{Dll3}\textit{neo} null allele. Our phenotypic analysis of \textit{Dll3}\textit{neo}/\textit{Dll3}\textit{neo} mutants shows that the developmental origins of the skeletal defects lie in delayed and irregular somite formation, which results in the perturbation of anteroposterior somite polarity. As the expression of \textit{Lfng}, \textit{Hes1}, \textit{Hes5} and \textit{Hey1} is disrupted in the presomitic mesoderm, we suggest that the somitic aberrations are founded in the disruption of the segmentation clock that intrinsically oscillates within presomitic mesoderm.

Key words: Notch signalling, Somite, Spondylocostal dysplasia, Pudgy, \textit{Dll3}, Mouse

INTRODUCTION

Notch signalling is an evolutionarily conserved mechanism used by metazoans to control the specification of cell fates through local interactions between cells (Artavanis-Tsakonas et al., 1999). As ligand and receptor are membrane associated, signalling is triggered by direct interaction of adjacent cells. In general, the Notch receptor is widely distributed within a cell population, while the ligand is restricted to a subset of cells (Fleming et al., 1990; Heitzler and Simpson, 1991; Vassil et al., 1987; Wharton et al., 1985). While several proteins participate in transmitting and regulating Notch signalling, a group of elements are defined as the core of this signalling pathway: in \textit{Drosophila}, Delta and Serrate are Notch ligands, the transcription factor Suppressor of Hairless [Su(H)] is the major downstream effector (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), and genes of the \textit{Enhancer of Split [E(Spl)]} locus (also transcription factors) are the primary targets of the pathway (Egan et al., 1998; Greenwald, 1998). Mammalian homologues have been identified for each of these core components and include \textit{Notch1}, \textit{Notch2}, \textit{Notch3} and \textit{Notch4} (Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1991; Weinmaster et al., 1992); Delta-like1 (\textit{Dll1}), \textit{Dll3} and \textit{Dll4} (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Shutter et al., 2000); Serrate homologues \textit{Jag1} and \textit{Jag2} (Lindsell et al., 1995; Shawber et al., 1996); \textit{Su(H)} homologue RBPjK (Furukawa et al., 1992; Schweisguth and Posakony, 1992) and Hairy and Enhancer of Split homologues \textit{Hes1}, \textit{Hes5} (Sasai et al., 1992; Takebayashi et al., 1995), \textit{Hey1} and \textit{Hey2} (also known as \textit{HRT}/\textit{Hesr}) (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999).

The Notch signalling pathway is deployed in three types of processes: lateral inhibition, lineage decisions and boundary formation (Bray, 1998). In vertebrates, somite segmentation relies on boundary formation in rostral presomitic mesoderm, coincident with expression of genes associated with Notch signalling (del Barco Barrantes et al., 1999). Accordingly, boundary formation with respect to somitogenesis commands...
considerable interest because, in mouse, core Notch signalling components (Notch1, Dll1, Dll3 and RBPjK) and signalling modifiers (Junctric fringe [LFng] and presenilin 1) are required for normal somite formation and anterior-posterior somite polarity (Conlon et al., 1995; Evrard et al., 1998; Hrabe de Angelis et al., 1997; Kusumi et al., 1998; Oka et al., 1995; Swiatek et al., 1994; Wong et al., 1997; Zhang and Gridley, 1998). In zebrafish, a mutation in deltaD is responsible for the after eight mutant (which makes only the first eight somites), demonstrating that Notch signalling is also required in this species (Holley et al., 2000).

In presomitic mesoderm, Notch signalling activity is not restricted to boundary formation, but also appears to be required at earlier (albeit interrelated) stages during the development of presomitic mesoderm (Pourquie, 2000). Presomitic mesoderm acquires a prepattern that distinguishes rostral presomitic mesoderm from caudal, and rostrally this culminates in segmentation with anteroposterior polarity being established in a single presomite unit. The periodicity with which this prepattern develops is postulated to require a 'segmentation clock' that oscillates in accordance with the formation of each new somite (Cooke, 1998; Cooke and Zeeman, 1976). Genes have been identified in chick (hairy-1), mouse (Lfng, Hes1, Hes7 and Hey2) and in zebrafish (her1, deltaC and deltaD) that produce transcripts that are seen to pass in a caudal to rostral direction (Aulehla and Johnson, 1999; Forsberg et al., 1998; Jiang et al., 2000; Bessho et al., 2001; Jouve et al., 2000; Leimeister et al., 2000; Leimeister et al., 1999; McGrew et al., 1998; Palmeirim et al., 1997). It is likely that Notch signalling is associated with the 'segmentation clock' because these genes are allied with Notch signalling: Fringe in Drosophila acts upstream of the pathway by modifying the response of Notch to ligand binding; deltaC and deltaD are ligands of Notch; and Hairy and Enhancer of Split homologues (chairy-1, Hes1, Hes7 and Hey2) are likely or proven downstream target genes of Notch signalling (Bessho et al., 2001; de la Pompa et al., 1997; del Barco Barrantes et al., 1999; Fleming et al., 1997; Holley et al., 2000; Jouve et al., 2000; Klein and Arias, 1998; Leimeister et al., 2000; Leimeister et al., 1999; Ohtsuka et al., 1999; Panin et al., 1997). This oscillatory pattern of gene expression consists of rostral and caudal expression components within the presomitic mesoderm. Characteristically, the rostral domain is condensed and corresponds to a half-somite segment, while the caudal domain is broader and moves rostrally from the caudal mesoderm. Characteristically, the rostral domain is condensed and corresponds to a half-somite segment, while the caudal expression components within the presomitic mesoderm. The most severe effects are seen in Dll1 and Jagged 1 (Jag1) expression is coincident in the posterior half of the forming somite, Dll3 expression is in the anterior half (del Barco Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsuadi et al., 1997; Zhang and Gridley, 1998), leading to the juxtaposition of Dll1/Jag1 co-expressing cells with Dll3-expressing cells across a forming somite boundary and within a forming somite. Genetic analysis reveals no somitic or vertebral defect in mouse Jag1 null mutants; however, butterfly vertebral do occur in Alagille Syndrome in which Jag1 is mutated (Krantz et al., 1997). By contrast, in Dll1 mutants the basic metameric unit within paraxial mesoderm is maintained albeit with a loss of anteroposterior polarity (del Barco Barrantes et al., 1999; Hrabe de Angelis et al., 1997; Xue et al., 1999). In the case of Dll3, pugdy mice have a highly disorganised vertebrocostal skeleton with delayed somite formation (Grunenberg, 1961; Kusumi et al., 1998). In humans, spondylocostal dysplasia (SCD) is characterised by similar vertebrocostal defects, and where SCD follows a recessive mode of inheritance, mutations have been reported in the DLL3 gene (Bulman et al., 2000).

We report the phenotypic analysis of a loss-of-function mutation in mouse Dll3 and demonstrate that this mutation affects the axial skeleton and components of the peripheral nervous system. The skeletal defects are severe and similar to those observed in cases of DLL3-dependent SCD in humans and Dll3/pugdy mice. In addition we show that the two mouse Dll3 mutant alleles, Dll3neo and Dll3pu, are functionally equivalent with respect to the skeletal defects. We use the null Dll3neo allele to show that the skeletal defects originate in aberrant somite formation, which are probably due to an altered ‘segmentation clock’ in presomitic mesoderm.

To understand how presomitic mesoderm prepatternning and segmentation culminates in somite formation, the role of core components of Notch signalling needs to be examined. In mouse, three Notch ligands are expressed in presomitic mesoderm. While Dll1 and Jagged 1 (Jag1) expression is coincident in the posterior half of the forming somite, Dll3 is expressed in the anterior half (del Barco Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsuadi et al., 1997). This oscillatory pattern of gene expression consists of rostral and caudal expression components within the presomitic mesoderm. While Dll1 and Jagged 1 (Jag1) expression is coincident in the posterior half of the forming somite, Dll3 is expressed in the anterior half (del Barco Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsuadi et al., 1997). This oscillatory pattern of gene expression consists of rostral and caudal expression components within the presomitic mesoderm. While Dll1 and Jagged 1 (Jag1) expression is coincident in the posterior half of the forming somite, Dll3 is expressed in the anterior half (del Barco Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsuadi et al., 1997; Zhang and Gridley, 1998), leading to the juxtaposition of Dll1/Jag1 co-expressing cells with Dll3-expressing cells across a forming somite boundary and within a forming somite. Genetic analysis reveals no somitic or vertebral defect in mouse Jag1 null mutants; however, butterfly vertebral do occur in Alagille Syndrome in which Jag1 is mutated (Krantz et al., 1997). By contrast, in Dll1 mutants the basic metameric unit within paraxial mesoderm is maintained albeit with a loss of anteroposterior polarity (del Barco Barrantes et al., 1999; Hrabe de Angelis et al., 1997; Xue et al., 1999). In the case of Dll3, pugdy mice have a highly disorganised vertebrocostal skeleton with delayed somite formation (Grunenberg, 1961; Kusumi et al., 1998). In humans, spondylocostal dysplasia (SCD) is characterised by similar vertebrocostal defects, and where SCD follows a recessive mode of inheritance, mutations have been reported in the DLL3 gene (Bulman et al., 2000).

MATERIALS AND METHODS

Targeting vector and generation of chimaeras

The Dll3 genomic clone was isolated from a 129sv library (Stratagene). Genomic DNA (2.5 kb and 3.4 kb) was cloned either side of PGK1-neomycin (Fig. 1A). This vector was linearised with XhoI and electroporated into CGR8 embryonic stem (ES) cells as described (Harrison et al., 1995). After double selection with G418 and gancyclovir, 800 ES cell clones were picked, expanded and frozen according to standard methods (Hogan et al., 1994). Homologous recombinants were identified following BamHI restriction and hybridisation with sequences located 5’ (Fig. 1A) and 3’ external to the recombination sites. Four targeted clones were identified and chimaeric males representing three clones were mated with C57BL6 females to establish F0 heterozygotes. These were crossed to C57BL6 mice and their progeny were intercrossed for phenotypic analysis. Results were pooled from the three distinct targeted Dll3/Dll3neo lines as individuals were phenotypically identical.

Genotyping Dll3, Dll3neo and Dll3pu alleles

Genotyping was performed by PCR or Southern blot (Fig. 1B; C, Hogan et al., 1994). PCR primers used to distinguish between Dll3 and Dll3neo were D3F (5’-tagtcaagctactcatgag-3’), D3R (5’-caatgaagagctatcag-3’) and PGK1 (5’-atgctcagctgctggg-3’). The Dll3neo allele was identified according to Kusumi et al. (1998).
Histology, in situ hybridisation and immunohistochemistry

For histology, embryos were fixed in Bouin’s fixative, dehydrated, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin as described (Kaufman, 1992). Whole-mount RNA in situ hybridisation was performed as described (Harrison et al., 1995). Probes for the following genes were used: **Dll3** (Dunwoodie et al., 1997), **Uncx4.1** (Mansouri et al., 1997), **Cer1** (Biben et al., 1998), **Hes1**, **Hes5** (Akazawa et al., 1992; Sasai et al., 1992), **Lfng** (Johnston et al., 1997) and **Mesp2** (Saga et al., 1997). pSPORT1-beta-spectrin2 (6412-8172bp) was linearised with SalI and antisense RNA generated using SP6 RNA polymerase. Skeletal preparations were performed at 14.5 dpc according to Jegalian and De Robertis (Jegalian and De Robertis, 1992). Whole-mount immunohistochemistry with anti-neurofilament monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank) was performed according to Mark et al. (Mark et al., 1993).

RESULTS

Targeted disruption of the **Dll3** gene and generation of null mutant mice

To engineer a **Dll3** null mutation a targeting vector was constructed deleting 5.4 kb of genomic sequence (Fig. 1A) including amino acids G135-S556 containing the DSL (Notch binding domain), all EGF repeats and the transmembrane domain (Dunwoodie et al., 1997; Kusumi et al., 1998). Mice heterozygous for the targeted allele (**Dll3neo**/) appeared normal. **Dll3** wild-type and targeted (**Dll3neo**/) alleles were distinguished by Southern blot or multiplex PCR analysis (Fig. 1B,C). Heterozygous (**Dll3/Dll3neo**) intercrosses resulted in the birth of homozygous (**Dll3neo/Dll3neo**) null mice. Genotypic analysis at post birthday (PBD) ten showed a deviation from the expected Mendelian ratio with 87% fewer **Dll3neo/Dll3neo** mutants present (Table 1). Further analysis indicated that **Dll3neo/Dll3neo** mutants were dying between birth and PBD10, as the genotype showed no deviation from the expected ratio during the prenatal period and at birth.

Skeletal defects in homozygous mutants

**Dll3neo/Dll3neo** mutants were easily identified because they had

<table>
<thead>
<tr>
<th>Table 1. Genotypes of mice resulting from heterozygous intercrosses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>Postnatal</td>
</tr>
<tr>
<td>Birth</td>
</tr>
<tr>
<td>Pre-natal</td>
</tr>
<tr>
<td>18.5 dpc</td>
</tr>
<tr>
<td>17.5 dpc</td>
</tr>
<tr>
<td>15.5 dpc</td>
</tr>
<tr>
<td>14.5 dpc</td>
</tr>
<tr>
<td>13.5 dpc</td>
</tr>
<tr>
<td>12.5 dpc</td>
</tr>
<tr>
<td>11.5 dpc</td>
</tr>
<tr>
<td>10.5 dpc</td>
</tr>
<tr>
<td>9.5 dpc</td>
</tr>
<tr>
<td>8.5 dpc</td>
</tr>
<tr>
<td>7.5 dpc</td>
</tr>
</tbody>
</table>

The genotype analysis combines data from three independent clones. Mice grouped into the postnatal category were between day 10 and day 20 at the time of tail biopsy. Ratios of genotypes were tested for goodness of fit to expected Mendelian segregation (1:2:1) by \( \chi^2 \) analysis, calculated with two degrees of freedom. dpc, days post coitum.
body was probably due to fewer vertebrae, and single vertebra showed more than one centre of ossification. In addition, the short tail in Dll3neo/Dll3neo individuals was due to the absence of approximately 20 coccygeal vertebrae.

Histological analysis at 13.5 dpc demonstrated irregularities in the peripheral nervous system (Fig. 3). In Dll3/Dll3neo embryos, the cartilage primordia of the vertebrae were regularly spaced like the dorsal root ganglia (Fig. 3A-C), while these were disorganised in Dll3neo/Dll3neo embryos (Fig. 3D-F). The cartilage primordium of the basioccipital bone appeared normal, with disorganisation apparent from the rostralmost vertebra (cervical 1) and extending along the entire length of the vertebral column (Fig. 2 and data not shown).

Neural crest cells arise without periodicity along the length of the neural tube; those that migrate ventrally condense to form ganglia (Larsen, 1997; Tosney, 1978; Weston, 1963). Similarly, axons of motoneurones that pass through a ventral root leave the neural tube along a broad front but they too condense to form discrete units. These ganglia and axons are located periodically along the length of the trunk despite the fact that they arise without periodicity from the neural tube. Periodicity is generated as the passage of neural crest and axons is restricted so that they migrate only through the anterior of the sclerotome (Stern and Keynes, 1987). This behaviour is not autonomous to the neural crest cells and axons, but rather is enforced by the sclerotome (Bronner Fraser, 1986; Rickmann et al., 1985; Teillet et al., 1987). Anti-neurofilament antibody confirmed the regular periodicity with which the spinal nerves and ganglia form in Dll3/Dll3neo embryos (Fig. 4A,B,F). Conversely, Dll3neo/Dll3neo embryos exhibited either lost or irregular periodic arrangement of ganglia and axons (Fig. 4C-E). In addition, the neural tube was often ‘kinked’ in Dll3neo/Dll3neo embryos (compare Fig. 4F with 4G).

**Somitogenesis is abnormal in Dll3neo/Dll3neo embryos**

Epithelial somites form from mesenchymal presomatic mesoderm in a rostrocaudal manner such that cells at the rostralmost part of the presomatic mesoderm will be the next to undergo a mesenchymal to epithelial transition to form a somite. Accordingly, cells at the caudal aspect of the presomatic mesoderm have only recently been recruited from the primitive streak (or tail bud) and so will form a somite only once they are located at the rostralmost position of the presomatic mesoderm. Epithelial somites were formed in Dll3neo/Dll3neo embryos; however, somite formation was delayed and the degree of condensation was reduced (Fig. 5). Using morphological landmarks and Mesp2 gene expression (Saga et al., 1997), the site of somite boundary formation was clearly identified in Dll3/Dll3neo embryos (Fig. 5A,B). In Dll3neo/Dll3neo embryos, the paraxial mesoderm was not organised into epithelial somites immediately rostral to this site (Fig. 5C-F). The extent of mesenchyme was inconsistent, suggesting that the delay in somite formation was variable between embryos. The expression of Mesp2 where the boundary should form in the Dll3neo/Dll3neo mutants demonstrates that this site is defined at the molecular level, despite the fact that a morphological transition was absent and that Mesp2 expression is independent of Dll3 function. Next, we examined whether somitogenesis was delayed from the
Delta3 is required for normal somitogenesis

Fig. 3. Skeletal dysplasia in Dll3neo/Dll3neo mutants leads to disorganisation of the peripheral nervous system. Parasagittal sections of 13.5 dpc Dll3/Dll3neo (A-C) and Dll3neo/Dll3neo (D-F) embryos. (A) Dorsal root ganglia and cartilage primordia of vertebrae are evenly spaced along the axis. (B) Enlargement of thoracic region shown in A. (C) The cartilage primordium of the basioccipital bone and the first two cervical vertebrae are clearly identifiable as is the rostralmost dorsal root ganglion which lies caudal to cervical vertebra 2. Note the even spacing of spinal nerves (dots) in (B,C). (D) Dorsal root ganglia are irregular in size and shape and are fused. This is evident in the thoracic region (E), where the arrangement of vertebrae and rib heads is also highly disorganised. Fused dorsal root ganglia are also evident in the cervical region (F) where the cartilage primordia of cervical vertebrae 1 and 2 are fused. Note the uneven distribution of spinal nerves (dots) in (E,F). Dorsal root ganglion (g), vertebra (v), head of rib (r), cervical vertebra (c), thoracic vertebra (t), basioccipital bone (b). Scale bar: 1.8 mm in A,D; 680 μm in B,C,E,F.

Fig. 4. Elements of the peripheral nervous system are disorganised in Dll3neo/Dll3neo mutants. Whole-mount immunohistochemistry with an anti-neurofilament antibody of Dll3/Dll3neo embryos (A,B,F) and Dll3neo/Dll3neo mutant embryos (C-E,G) at 10.5-11.5 dpc. Lateral view (A-E) and dorsal view (F,G). (A) Dorsal root ganglia (drg), spinal nerve (sn) and sensory chain ganglia (scg) are evenly spaced. (B) The region between the fore and hind limbs of (A) is marked with a line (anterior towards the top). Lines dorsal and ventral to the somites mark individual somitic segments and show that ventral spinal axons pass exclusively through the anterior of the somite segment. (C) drg, sn and scg are unevenly spaced. (D) The region between the fore and hind limbs of (C) is marked with a line (anterior towards the top). Lines mark out individual somitic segments and show that the spinal axons pass through the anterior, posterior or central part of the somite segment. (E) Dll3neo/Dll3neo mutant embryo (11.5 dpc). The disarray of spinal axons and scg is more severe than in (C). (F) Dorsal view of A shows a straight neural tube, while in G the same view of E indicates that the neural tube is ‘kinked’ (anterior towards the top). Scale bar: 730 μm in A,C,E; 150 μm in B,D; 365 μm in F,G.
onset of somite formation. Physical boundaries were evident between somites in Dll3/Dll3 and Dll3/Dll3neo embryos (Fig. 5G,H), but were not detected in Dll3neo/Dll3neo mutants (Fig. 5I-L). Although intersomitic boundaries were not observed metameric units were evident as reduced epithelialisation of the cytoskeletal protein β-spectrin2. Notwithstanding the delay and irregularity associated with somite formation in Dll3neo/Dll3neo embryos, the band of Mesp2 expression is in an equivalent position to that in A. (B,D,F) Higher magnifications of A.C.E. (G-L) Ventral view of 8.5 dpc embryos after whole-mount in situ hybridisation with β-spectrin 2. The arrow indicates where the next somite boundary will form. (G,H) Epithelialisation of mesenchyme to form somites is evident and marked by distinct β-spectrin 2 gene expression in the centre of the somite in Dll3/Dll3neo embryos.

Delayed and irregular somite formation could explain the skeletal dysplasia in Dll3neo/Dll3neo mutants. However, as each vertebra is derived from the anterior of one somite and the posterior of an adjacent somite (Bagnall et al., 1988; Bagnall et al., 1989; Huang et al., 1996; Stern and Keynes, 1987), vertebral development is also dependent upon anteroposterior somite identity being clearly defined. Uncx4.1 expression marks the posterior nascent somite and later, the posterior lateral sclerotome (Fig. 6C) (Mansouri et al., 1997; Neidhardt, 1997) and was used to examine somite polarity. Expression in posterior epithelial somites was clearly evident in caudal somites dissected from Dll3/Dll3neo embryos (Fig. 6F). By contrast, Uncx4.1 was mostly (5/8) expressed in a continuous domain in the paraxial mesoderm of Dll3neo/Dll3neo embryos, the remainder (3/8) showing periodic, but not exclusively posterior, expression (Fig. 6D,E). In addition, epithelial somites were not discernable in paraxial mesoderm dissected from Dll3neo/Dll3neo mutants (compare Fig. 6F with 6G). As paraxial mesoderm of Dll3neo/Dll3neo embryos displays posterior character (albeit disorganised), we examined whether anterior character was apparent. Mouse Cer1 was expressed in stripes in the anterior presomitic mesoderm and the anterior of nascent somites in Dll3/Dll3 (12/12) and Dll3/Dll3neo (25/25) embryos (Biben et al., 1998; Fig. 6H). Dll3neo/Dll3neo mutants also expressed Cer1 in paraxial mesoderm, demonstrating the presence of anterior character; however, as for Uncx4.1, expression lacked periodicity (11/11; Fig. 6I-K).
expression at 10.5 dpc revealed four distinct patterns of
et al., 1999; Takebayashi et al., 1995). Analysis of gene
forming somite (de la Pompa et al., 1997; del Barco Barrantes
band in rostral presomitic mesoderm in the posterior half of the
/Dll3 neo embryos and rostral expression was diffuse. Uncx4.1 expression shows clear periodicity in
Dll3 neo Dll3
expression is reduced in
Dll3 neo/Dll3
mutants. Periodic expression is partially retained in
some embryos (D) or lost (E). (G) Trunk paraxial mesoderm
dissected from D; expression appears periodic but is not restricted to
the posterior somite (note the lack of epithelial structure). Dorsal
view showing Cer1 expression in the tail region at 10.5 dpc of
Dll3/Dll3
(H) and Dll3 neo/Dll3
embryos (I-K). (H) Cer1 is expressed anteriorly in the presumptive somite and the nascent
somite. Arrows mark the site of the most recently formed somite boundary and vertical bars show expression in the anterior of the
nascent somite. (I-K) Cer1 is expressed in a broad domain (vertical bar) as the bands of expression are missing. Scale bar: 125 μm in
A,B; 300 μm in C-E; 60 μm in F,G; 120 μm (H-K).

Defining downstream effectors of Dll3-mediated
Notch signalling
As Hes5, Hes1 and Hey1 have been identified as genes responsive to Notch signalling, their expression was examined in
Dll3 neo/Dll3
embryos. Hes5 is normally expressed as a band in rostral presomitic mesoderm in the posterior half of the forming somite (de la Pompa et al., 1997; del Barco Barrantes et al., 1999; Takebayashi et al., 1995). Analysis of gene
expression at 10.5 dpc revealed four distinct patterns of
expression in presomitic mesoderm of Dll3/Dll3
and Dll3/Dll3 neo embryos. These patterns of expression indicate that Hes5, like closely related Hes1 and Hes7, is dynamically expressed in the presomitic mesoderm (Fig. 7A-D) (Bessho et al., 2001; Jouve et al., 2000). By contrast, at 10.5 dpc, Hes5 expression was not detected in presomitic mesoderm in eight
out of nine Dll3neo/Dll3neo mutant embryos (Fig. 7E,F). In one embryo, a single faint band was detected in rostral presomitic mesoderm (data not shown). Similarly, at 9.5 dpc, Hes5 expression was not detected in presomitic mesoderm in six out of six Dll3neo/Dll3neo mutant embryos (data not shown). Hes1 is expressed in the caudal half of nascent somites and dynamically in presomitic mesoderm (Jouve et al., 2000). In the presomitic mesoderm of Dll3/Dll3 and Dll3/Dll3neo embryos, dynamic expression is detected as a broad caudal domain that appears to narrow as it moves rostrally to form a tight band coincident with somite formation (Fig. 7G-I). Rostral expression is evident alone or in combination with this caudal domain of expression, depending upon the stage of the cycle. At 10.5 dpc Dll3/Dll3 embryos exhibited either rostral alone (3/10) or rostral and caudal domains (7/10) of Hes1 expression. Similarly Hes1 expression was detected as a single rostral domain (6/11) or with rostral and caudal domains (5/11) in Dll3/Dll3neo. This pattern of expression was not evident in Dll3neo/Dll3neo mutant embryos because, in ten out of ten, only a single narrow band of Hes1 expression was detected in the rostral presomitic mesoderm (Fig. 7J,K). In addition, no Hes1 expression was detected in the somites where normally it is detected caudally (compare Fig. 7G-I with 7J,K). Hey1 is expressed in the caudal half of the most recently formed somite and in a band in the rostral presomitic mesoderm which narrows as a somite forms (Kokubo et al., 1999; Leimeister et al., 1999). Hey1 expression in wild-type (Dll3/Dll3) and heterozygous (Dll3/Dll3neo) embryos reflects this pattern of expression with 10/24 embryos the same as Fig. 7L and 14/24 the same as Fig. 7M. By contrast, in 18 out of 18 Dll3neo/Dll3neo mutant embryos, Hey1 expression appeared static because only a single band of expression was detected in the rostral presomitic mesoderm (Fig. 7N). In addition, expression normally present in the caudal somites was not detectable in most mutants (compare Fig. 7L,M with 7N). In summary, these data demonstrate that Dll3 is required for the normal expression of Hes5, Hes1 and Hey1 in presomitic mesoderm.

**DISCUSSION**

The mutant Dll3 alleles; Dll3neo, Dll3pu and DLL3-SCD are functionally equivalent with respect to skeletal dysplasia

Uncertainty has surrounded the mouse Dll3pu allele because it is unclear whether this allele is null. Although a four base deletion is predicted to generate a stop codon in exon 3 (N-terminal to the DSL that lies in exon 4) (Kusumi et al., 1998), it is possible that splicing around the deletion occurs and some functional Dll3 protein is produced. As we have been unable to generate anti-Dll3 antibodies this scenario has remained untested; however, our genetic complementation studies indicate that this is unlikely to be the case because Dll3neo and Dll3pu are equivalent alleles with respect to skeletal dysplasia (Fig. 8). We observed that Dll3neo/Dll3neo individuals are slightly more severely affected than Dll3pu/Dll3pu, but this is likely to be due to differences in mouse strain (Dll3neo/Dll3neo embryos are 129Ola/C57BL6, whereas the Dll3pu/Dll3pu embryos are C3H/He/C57BL6). In humans, sequence analysis has defined three SCD-associated DLL3 mutations. SD1 contains a five base insertion, SD2 a two base deletion and SD3 a missense mutation in EGF repeat number 5 (Bulman et al., 2000). The effect of the missense mutation on protein function is unknown; however, SD1 and SD2 mutations generate truncated proteins that are not membrane tethered but could interact with Notch because the DSL (Notch-binding region) is present either in full (SD2) or in part (SD1). This raises the possibility that these mutants do not represent null alleles,
because soluble DLL3 forms could interact with Notch and either activate the receptor without being tethered to a neighbouring cell or prevent another ligand from binding Notch. However, as the Dll3 neo null mutation has very similar phenotypic effects on the development of the axial skeleton, we suggest that each of the human SCD alleles are likely to represent null mutations.

The developmental origin of skeletal defects associated with SCD lie in the disruption of the segmentation clock within the presomitic mesoderm

Generation of the Dll3 neo mutant mouse lines has allowed us to examine the developmental origins of the skeletal defects presented in SCD. The core SCD phenotype is characterised by multiple hemi-vertebrae with rib fusions and deletions. The developmental origins of this phenotype reside in aberrant developmental origins of this phenotype reside in the loss of somite formation – a defect that appears grounded in the loss of the oscillatory mechanism that drives the regular periodicity with which somites are formed. The molecular analysis of somite formation is a defect that appears grounded in the loss of Notch signalling, whose normal expression in the presomitic mesoderm is dependent upon Dll3 function. These include Lfng, Hes5, Hes1 and Hey1, and therefore these are candidate genes responsible for cases of SCD that show no link to DLL3/19q13.

Are Dll1 and Dll3 distinct ligands of Notch in paraxial mesoderm?

Both Dll1 and Dll3 are required for normal somite formation and correct specification of anteroposterior polarity within the presomitic mesoderm (Gruneberg, 1961; Hrabe de Angelis et al., 1997; Kusumi et al., 1998) (Figs 5, 6). As Dll1 and Dll3 are both ligands of Notch, what evidence is there that they are distinct ligands that elicit different downstream responses? This study shows that markers of anterior (Cer1) and posterior (Uncx4.1) somite identity are expressed at normal levels in the absence of Dll3, but that the periodic expression of Uncx4.1 and Cer1, which is characteristic of anteroposterior polarity, is lost (Fig. 6 and summarised in Fig. 9). By contrast, anteroposterior identity is lost in Dll1 mutants, as Uncx4.1 is not detected, while Cer1 (and EphA4 another marker of anterior) are severely downregulated (del Barco Barrantes et al., 1999) (Fig. 9). In addition, we present evidence to suggest that Dll1 and Dll3 elicit distinct responses from genes associated with Notch signalling. For example, a loss-of-function mutation in Dll1 results in severely downregulated (and largely undetected) expression of Lfng, Hes5, Hes1, Hey1, Mesp1 and Mesp2 in presomitic mesoderm (del Barco Barrantes et al., 1999; Jouve et al., 2000; Kokubo et al., 1999) (Fig. 9). By contrast, with the exception of Hes5, the expression of each of these genes is readily detected in presomitic mesoderm of Dll3 null mutants (Figs 5-7, 9; Mesp1 was not examined). That Dll1 and Dll3 may be distinct is further supported by the fact that Dll3 is a highly divergent Delta homologue (Dunwoodie et al., 1997) and has only 18% identity to the Notch binding DSL of Dll1, compared with the 51% identity between Dll4 and Dll1. It is, however, possible that when Dll1 and Dll3 mutants are compared that some of the observed differences in gene expression do not indicate discrete functions for these ligands but rather reflect the possibility that Dll1 and Dll3 perform the same function and affect the expression of specific genes to different extents. As Dll1 and Dll3 are differentially expressed in presumptive and nascent somites, this issue could best be addressed by placing the Dll1 cDNA under the regulatory control of Dll3 or vice versa using a cDNA ‘knock-in’ approach.

Oscillatory gene expression in the presomitic mesoderm

Genes expressed in an oscillatory manner in the presomitic mesoderm are likely to hold the key to our understanding of how exactly Notch signalling controls somitogenesis. We show for the first time that Hes5 exhibits a number of distinct patterns of expression in the presomitic mesoderm. This suggests that Hes5, like Hes1 and Hes7, is expressed under the control of oscillatory stimuli. The regulatory parameters that control the dynamic expression of these ‘clock’ genes in presomitic mesoderm are unknown. We present data to indicate that the rostral and caudal expression components of these genes are differentially controlled by Dll3 and Dll1. Expression of Lfng is severely downregulated (del Barco Barrantes et al., 1999) and Hes1 is not detected (Jouve et al., 2000) in the presomitic mesoderm of Dll1 null mutants, but in Dll3 neo null mutants, only the caudal expression component is lost with the rostral band clearly evident (Figs 6, 7, 9). These differences could simply be due to different levels of gene expression; however, this does not appear to be the case because the rostral and caudal expression domains of Hes1 in...
the presomitic mesoderm normally occur at equal levels and so the loss of just the caudal domain inDll3 mutants cannot be explained by an overall reduction in expression levels. In the case ofLfng, even though the rostral domain is normally expressed at levels higher than that seen caudally, rostral expression in Dll3 mutants was readily detectable and no caudal expression was ever observed, even under extensive periods of staining. This suggests that the rostral and caudal components that drive expression of oscillatory genes such asHes1 and Lfng in the presomitic mesoderm are independently controlled and that both components requireDll1, but only the caudal component requiresDll3.

A comparison ofDll3 and deltaD mutants

The mutant phenotype ofDll3 resembles that of deltaD (after eight) in zebrafish at a number of levels. First, in both mutants somite formation occurs in the first instance with what appears to be the correct periodicity. This is followed by delayed somite formation in Dll3 mutants, and lack of somite formation in deltaD mutants. However, even though metamerism was apparent in Dll3 mutants, borders between somites were not evident and condensation of paraxial mesoderm into somites was reduced compared with wild type (Fig. 5). That somitogenesis is not completely normal is supported by the fact that the vertebrae caudal to and including cervical 1 (which is comprised of the anterior part of the fifth formed somite) was not properly formed. Second, marker gene expression indicates that paraxial mesoderm in Dll3 and deltaD mutants has both anterior (Dll3 – Cer1 and Mesp2, deltaD – mesp-a, Epha4, fgf8 and deltaD) and posterior (Dll3 – Unc4x.1 and Cited1; deltaD – ephrin-B2 and MyoD) character (Fig. 6, data not shown) (Durbin et al., 2000). Third, although paraxial mesoderm has anterior and posterior identity in both mutants, like cells are not grouped and spaced periodically (Fig. 6) (Durbin et al., 2000). Finally, both mutants show disrupted expression of genes expressed in a cyclical manner in the presomitic mesoderm. In Dll3 mutants, Lfng, Hes1 and Hes5 expression is disrupted, while in deltaD, her1 expression is disrupted. As mutant expression of Lfng, Hes1 and her1 consists of what appears to be a static band in the rostral presomitic mesoderm in the absence of caudal expression, there is potentially a common mechanism that is responsible for the oscillatory gene expression in presomitic mesoderm (Fig. 7) (Holley et al., 2000).

We thank A. Stewart and E. Grigorieva for excellent technical assistance. Clones were provided by Y. Saga (Mesp2), R. Johnston (Lunatic Fringe), B. Hermann (Unc4x.1), R. Harvey (Cer-1), M. Gessler (Hey1) and C. Lobe (Hes1, Hes5). This work was funded by NHMRC project grant #142006 (SLD), the National Science Foundation and the March of Dimes (RAC) and Core MRC programme support (RSPB).

REFERENCES


Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe
in the dorsal compartment of the Drosophila wing imaginal disc. 

Development 124, 2973-2981.


Curr. Biol. 8, 1027-1030.


Cell 69, 1191-1197.


Acta Anat. 125, 76-82.


Genes Dev. 12, 1751-1762.


Genetic Res. 2, 384-393.


Development 121, 2479-2489.


Cell 64, 1083-1092.


Genes Dev. 14, 1678-1690.


Nature 386, 716-721.


Dev. Biol. 227, 91-103.


Dev. Biol. 227, 91-103.


Cell 80, 909-917.


Dev. Dyn. 210, 53-65.


Development 119, 319-338.


Dev. Biol. 205, 799-802.


Development 121, 2479-2489.


