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

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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 (*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 *Dll3*-dependent pudgy
mutant in mouse and with spondylocostal dysplasia (MIM
277300) in humans. This study demonstrates that *Dll3neo* and
*Dll3pu* are functionally equivalent alleles with respect to the
skeletal dysplasia, and we suggest that the three human
*DLL3* mutations associated with spondylocostal dysplasia
are also functionally equivalent to the *Dll3neo* null allele. Our
phenotypic analysis of *Dll3neo/Dll3neo* 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 *Lfng, Hes1, Hes5* and *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, *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 *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
*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 *Notch1*, *Notch2*, *Notch3*
and *Notch4* (Lardelli et al., 1994; Uyttendaele et al., 1996;
Weinmaster et al., 1991; Weinmaster et al., 1992); Delta-like1
(*Dll1*), *Dll3* and *Dll4* (Bettenhausen et al., 1995; Dunwoodie
et al., 1997; Shutter et al., 2000); Serrate homologues *Jag1* and
*Jag2* (Lindsell et al., 1995; Shawber et al., 1996); Su(H)
homologue RBPjK (Furukawa et al., 1992; Schweisguth and
Posakony, 1992) and Hair and Enhancer of Split homologues
*Hes1, Hes5* (Sasai et al., 1992; Takebayashi et al., 1995), *Hey1*
and *Hey2* (also known as *HRT/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 [lunatic fringe (Lfg) 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 (hair-l), mouse (Lfg, 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 (hair-l, 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 presomitic mesoderm. In cases where the core components of Notch signalling have been targeted, null mutant embryos show disrupted oscillatory gene expression 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 is expressed in the anterior half (del Barco Barrantes et al., 1999; Dunwoodie et al., 1997; Mitsiadis 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 vertebrae 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 (Gruneberg, 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 mouseDll3 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 nullDll3neo allele to show that the skeletal defects originate in aberrant somite formation, which are probably due to an altered ‘segmentation clock’ in presomitic mesoderm.

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 C57BL6 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 recombinant 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'-tatgcaagactccatcattgagcc-3'), D3R (5'-atgctccagactgccttggg-3'), Jag1 (5'-atgctccagactgccttggg-3'), Jag1 (5'-atgctccagactgccttggg-3') and PGK1 (5'-atgctccagactgccttggg-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 Haematoxylin-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 1. Genotypes of mice resulting from heterozygous intercrosses](image_url)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>Dll3/Dll3</th>
<th>Dll3/Dll3neo</th>
<th>Dll3neo/Dll3neo</th>
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<tr>
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<td>84</td>
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<tr>
<td>Dll3/Dll3neo</td>
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<td>120</td>
<td>36</td>
<td>0.021</td>
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</tr>
<tr>
<td>Dll3neo/Dll3neo</td>
<td>58</td>
<td>120</td>
<td>36</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>Dll3/Dll3</td>
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<td>575</td>
<td>282</td>
<td>0.537</td>
<td></td>
</tr>
<tr>
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<td>0.481</td>
</tr>
<tr>
<td>Dll3/Dll3neo</td>
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<td>2</td>
<td>0.982</td>
</tr>
<tr>
<td>Dll3/Dll3neo</td>
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<td>2</td>
<td>3</td>
<td>2</td>
<td>0.982</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>2</td>
<td>0.982</td>
</tr>
<tr>
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<td>3</td>
<td>2</td>
<td>0.982</td>
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<td>2</td>
<td>3</td>
<td>2</td>
<td>0.982</td>
</tr>
<tr>
<td>Dll3/Dll3neo</td>
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<td>3</td>
<td>2</td>
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<td>2</td>
<td>3</td>
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<td>2</td>
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<td>3</td>
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<td>2</td>
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<td>0.982</td>
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</tbody>
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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.
a shortened body (40% reduced) and a short tail (Fig. 2A). This defect was completely penetrant and was apparent in preskeletal cartilage in embryos at 14.5 dpc (Fig. 2B). Skeletal disorganisation extended from the most rostral vertebra (cervical 1) along the length of the vertebral column. The vertebral arches were highly disorganised with ribs sometimes fused or absent (compare Fig. 2C with 2D). Shortening of the body was probably due to fewer vertebrae, and single vertebra showed more than one centre of ossification. In addition, the short tail in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ 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 $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos, the cartilage primordia of the vertebrae were regularly spaced like the dorsal root ganglia (Fig. 3A-C), while these were disorganised in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ 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 $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos (Fig. 4A,B,F). Conversely, $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos exhibited either lost or irregular periodic arrangement of ganglia and axons (Fig. 4C-E). In addition, the neural tube was often ‘kinked’ in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos (compare Fig. 4F with 4G).

**Somitogenesis is abnormal in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos**

Epithelial somites form from mesenchymal presomitic mesoderm in a rostrocaudal manner such that cells at the rostralmost part of the presomitic mesoderm will be the next to undergo a mesenchymal to epithelial transition to form a somite. Accordingly, cells at the caudal aspect of the presomitic 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 presomitic mesoderm. Epithelial somites were formed in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos; however, somite formation was delayed and the degree of condensation was reduced (Fig. 5). Using morphological landmarks and $\text{Mesp2}$ gene expression (Saga et al., 1997), the site of somite boundary formation was clearly identified in $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ embryos (Fig. 5A,B). In $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ 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 $\text{Mesp2}$ where the boundary should form in the $\text{Dll3}^{\text{neo}}/\text{Dll3}^{\text{neo}}$ mutants demonstrates that this site is defined at the molecular level, despite the fact that a morphological transition was absent and that $\text{Mesp2}$ expression is independent of $\text{Dll3}$ function. Next, we examined whether somitogenesis was delayed from the
Delta3 is required for normal somitogenesis

Fig. 3. Skeletal dysplasia in Dll3\textsuperscript{neo}/Dll3\textsuperscript{neo} mutants leads to disorganisation of the peripheral nervous system. Parasagittal sections of 13.5 dpc Dll3/Dll3\textsuperscript{neo} (A-C) and Dll3\textsuperscript{neo}/Dll3\textsuperscript{neo} (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\,\mu m in B,C,E,F.

Fig. 4. Elements of the peripheral nervous system are disorganised in Dll3\textsuperscript{neo}/Dll3\textsuperscript{neo} mutants. Whole-mount immunohistochemistry with an antineurofilament antibody of Dll3/Dll3\textsuperscript{neo} embryos (A,B,F) and Dll3\textsuperscript{neo}/Dll3\textsuperscript{neo} 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) Dll3\textsuperscript{neo}/Dll3\textsuperscript{neo} 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\,\mu m in A,C,E; 150\,\mu m in B,D; 365\,\mu m in F,G.
Fig. 5. Somitogenesis is delayed and irregular with reduced mesenchymal condensation in Dll3neo/Dll3neo mutants. (A-F) Lateral view of the caudal part of 9.5 dpc embryos after RNA whole-mount in situ hybridisation with Mesp2. The black line (A-D) indicates the position of the last formed somite boundary and Mesp2 expression lies just caudal to the somite boundary that is next to form in (A,B). (A) Regularly sized and spaced epithelial somites are present in Dll3/Dll3neo embryos. The distance between the band of Mesp2 expression and the last formed somite is equivalent to one somite width. (C) Irregularly sized epithelial somites are formed in this Dll3neo/Dll3neo embryo. The distance between the band of Mesp2 expression and the last formed somite is equivalent to three somite widths. (E) No epithelial somites were detected in this Dll3neo/Dll3neo embryo. 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. (I-L) Epithelialisation of mesenchyme is poor in Dll3neo/Dll3neo mutants, with diffuse β-spectrin 2 expression. (H,J,L) Higher magnifications of G,I,K. Scale bar: 200 μm in A,C,E; 100 μm in B,D,F; 160 μm in G,I,K; 80 μm in H,J,L.

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 metamerically units were evident as reduced epithelialisation of paraxial mesoderm was detected by the less condensed expression of the cytoskeletal protein β-spectrin 2. Notwithstanding the delay and irregularity associated with somite formation in Dll3neo/Dll3neo mutants, they differentiated into muscle, dermis and skeleton, indicating that dorsoventral pattern was established and cellular differentiation achieved (Fig. 3 and data not shown).

In order that the specified number of evenly sized somites are generated, a boundary must form at regular time intervals in the rostral presomitic mesoderm, and this most probably requires the dynamic action of Lfng in the presomitic mesoderm (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). Lfng transcripts are localised to one or two bands adjacent to the forming somite boundary in the rostral presomitic mesoderm, and in a caudal dynamic domain. A reiterative pattern is produced which is completed in approximately 2 hours in the mouse, roughly the period of time required to generate a somite (Goedbloed and Smits-van Prooije, 1986; Tam and Tan, 1992). Given that the somite boundary does not form at the usual site in Dll3neo/Dll3neo mutants, we examined Lfng expression. In Dll3/Dll3 and Dll3/Dll2neo embryos, Lfng expression was detected in both rostral and caudal presomitic mesoderm (12/15) in patterns consistent with those reported in mouse (Aulehla and Johnson, 1999; Forsberg et al., 1998) (Fig. 6A). By contrast, a rostral domain of expression was apparent in the absence of a caudally located expression domain in Dll3neo/Dll3neo mutants (10/10) (Fig. 6B).

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 Dll2neo/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 Cerl was expressed in stripes in the anterior presomitic mesoderm and the anterior of nascent somites in Dll3/Dll3 (12/12) and Dll3/Dll2neo (25/25) embryos (Biben et al., 1998; Fig. 6H). Dll2neo/Dll3neo mutants also expressed Cerl in paraxial mesoderm, demonstrating the presence of anterior character; however, as for Uncx4.1, expression lacked periodicity (11/11; Fig. 6I-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 Dll3neo/Dll3neo 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
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 Dll3neo 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 Dll3neo 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 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 Dll3neo/Dll3neo embryos identifies genes associated with 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 Dll3neo 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

![Fig. 9. A comparison of gene expression in paraxial mesoderm of normal and Delta mutants. Black and grey areas represent localisation of transcript and the wavy line represents dynamic gene expression. The most recently formed somite is SI, the forming somite is S0, and the block of presomitic mesoderm cells of one somite length (caudal to S0) is S-1 according to (Dale and Pourquie, 2000; Oraldi, 1993). Gene expression was determined by RNA in situ hybridisation. This study examined expression in wild-type and Dll3 mutant embryos. Wild-type expression patterns were in accordance with those previously reported: Mesp2 (Saga et al., 1996), Cer1 (Biben et al., 1998), Uncx4.1 (Mansouri et al., 1997; Neidhardt, 1997), Lfng (Forsberg et al., 1998; Johnston et al., 1997), Hes1 (Jouve et al., 2000), Dll1 (Bettenhausen et al., 1995; Dunwoodie et al., 1997) and Dll3 (Dunwoodie et al., 1997). For Hes5, we identified four distinct patterns of expression in presomitic mesoderm (Fig. 7). Gene expression in Dll1 mutants is based on previous reports (del Barco Barrantes et al., 1999; Jouve et al., 2000). The levels of expression of Dll1 in Dll1 mutant embryos was low with diffuse boundaries and is indicated by grey shading.](image-url)
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 of Lfng, even though the rostral domain is normally expressed at levels higher than that seen caudally, rostral expression inDll3 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 as HesL 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 ofdeltaD (after eighth) 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 inDll3 mutants, and lack of somite formation indeltaD mutants. However, even though metamersism was apparent inDll3 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 inDll3 anddeltaD mutants has both anterior (Dll3–CerL and Mesp2; deltaD–mesp-a, Epha4, fgf8 anddeltaD) and posterior (Dll3–Unc44.1 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. InDll3 mutants, Lfng, HesL and Hes5 expression is disrupted, while indeltaD, herL expression is disrupted. As mutant expression ofLfng, HesL and herL 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 (Unc44.1), R. Harvey (Cer-L), M. Gessler (Hey1) and C. Lobe (Hes1, Hes5). This work was funded by NHRMC project grant #142006 (SLD), the National Science Foundation and the March of Dimes (RAC) and Core MRC programme support (RSPB).

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