Convergent extension, planar-cell-polarity signalling and initiation of mouse neural tube closure

Patricia Ybot-Gonzalez*, Dawn Savery*, Dianne Gerrelli*, Massimo Signore, Claire E. Mitchell†, Clare H. Faux‡, Nicholas D. E. Greene and Andrew J. Copp§

Planar-cell-polarity (PCP) signalling is necessary for initiation of neural tube closure in higher vertebrates. In mice with PCP gene mutations, a broad embryonic midline prevents the onset of neurulation through wide spacing of the neural folds. In order to evaluate the role of convergent extension in this defect, we vitally labelled the midline of loop-tail (Lp) embryos mutant for the PCP gene Vangl2. Injection of Dil into the node, and electroporation of a GFP expression vector into the midline neural plate, revealed defective convergent extension in both axial mesoderm and neuroepithelium, before the onset of neurulation. Chimeras containing both wild-type and Lp-mutant cells exhibited mainly wild-type cells in the midline neural plate and notochordal plate, consistent with a cell-autonomous disturbance of convergent extension. Inhibitor studies in whole-embryo culture demonstrated a requirement for signalling via RhoA-Rho kinase, but not jun N-terminal kinase, in convergent extension and the onset of neural tube closure. These findings identify a cell-autonomous defect of convergent extension, requiring PCP signalling via RhoA-Rho kinase, during the development of severe neural tube defects in the mouse.

KEY WORDS: Mouse, Neurulation, Morphogenesis, Neural tube defects, Convergent extension, Planar cell polarity, Chimeras, Embryo culture

INTRODUCTION

Shaping of the vertebrate embryo during the late stages of gastrulation converts the elliptical gastrula into the keyhole-shaped neurula. This shaping results largely from the cell movements of convergent extension that involve lateral-to-medial displacement of cells in the plane of the ectoderm, and in the underlying mesoderm (Keller, 2002). Cell intercalation in the midline leads to rostrocaudal extension of the body axis. An essential role in convergent extension has been assigned to the planar-cell-polarity (PCP) pathway, a highly conserved, non-canonical Wnt-frizzled-dishevelled signalling cascade. PCP signalling plays a key role in establishing and maintaining polarity in the plane of epithelia in Drosophila, and in epithelial and non-epithelial tissues in vertebrates (Strutt, 2003; Klein and Mlodzik, 2005).

Both fish and amphibian embryos require an intact PCP pathway for postgastrulation shaping (Wallingford and Harland, 2001; Park and Moon, 2002; Darken et al., 2002; Jessen et al., 2002). Moreover, in Xenopus, PCP-dependent convergent extension in the midline is required for neural tube formation following gastrulation (Wallingford and Harland, 2002; Takeuchi et al., 2003). In its absence, the neural folds are spaced widely apart, precluding the apposition and fusion that is required for neural tube closure (Wallingford and Harland, 2002). A similar failure of initiation of neurulation occurs in several mouse mutants: loop-tail (Lp), circletail (Crc), crash (Crsh), protein tyrosine kinase 7 (Ptk7), dishevelled 1 (Dvl1) and Dvl2 double mutants, and frizzled 3 (Fz3) and Frz6 double mutants (Greene et al., 1998; Murdoch et al., 2001b; Hamblet et al., 2002; Lu et al., 2004; Wang et al., 2006b). This yields the severe defect craniorachischisis (CRN), in which the neural tube remains open from midbrain to low spine (Botto et al., 1999; Copp et al., 1994). CRN comprises up to 10% of human neural tube defects (Moore et al., 1997), and seems likely to result from failure of the initial event of neural tube closure, as in the mouse (Kirillova et al., 2000).

All six of the gene loci associated with CRN in the mouse adversely affect PCP function. The Lp mouse is mutant for Vangl2 (Kibar et al., 2001; Murdoch et al., 2001a), the homologue of Drosophila strabismus and van gogh, which encodes a protein that binds directly to dishevelled, participating in its recruitment to the cell membrane (Bastock et al., 2003; Torban et al., 2004). Crash mice are mutant for Celsr1 (Curtin et al., 2003), the homologue of Drosophila starry night (also known as flamingo), which plays a role in assembling the PCP protein complex, at least in Drosophila (Strutt, 2003). Loss of function of two of the three dishevelled genes (Dsh-1 and -2), and two of the six frizzled genes (Frz-3 and -6), also leads to CRN (Hamblet et al., 2002; Wang et al., 2006b). Moreover, mutation of Scrib (also known as Scrb1) in circletail mice (Murdoch et al., 2003) and a gene-trap mutation of the tyrosine kinase Ptk7 (Lu et al., 2004) generate a CRN phenotype. Although not directly implicated in the PCP molecular pathway, both Scrb1 and Ptk7 interact genetically with the other mouse CRN genes (Murdoch et al., 2001b; Lu et al., 2004) and produce a planar-polarity phenotype in the inner ear (Montcouquiol et al., 2003; Lu et al., 2004). It is striking, therefore, that of the more than 100 genes necessary for neural tube closure in the mouse, all of those with a CRN phenotype disrupt PCP signalling (Copp et al., 2003).

A defect of convergent extension seems likely to precede the failure of neural tube closure in mouse PCP mutants, in view of the widely spaced neural folds described in Lp, Crc, Dvl1;Dvl2 and Ptk7 homozygotes (Greene et al., 1998; Murdoch et al., 2003; Lu et al., 2004; Wang et al., 2006a), and the finding of an abnormally short and wide body axis in Lp/Lp and Dvl1;Dvl2 embryos during late
gastulation (Wang et al., 2006a). To date, however, a direct analysis of convergent extension in mouse PCP mutants has not been reported. In the present study, we vitally labelled pre-neurulation stage embryos in order to study convergent extension and found that midline extension is defective in both the axial mesoderm and the neural plate of Lp/Lp mutants. Analysis of +/+ ↔ Lp/Lp chimeras suggested a cell-autonomous defect in the convergence of Lp/Lp cells towards the neural plate midline along the entire body axis, as well as in cells recently emerged from the node, the source of axial mesoderm and midline neural plate in the mouse (Sausedo and Schoenwolf, 1994; Sulik et al., 1994). Among possible signalling pathways downstream of the PCP genes, we found function of RhoA-Rho kinase (ROCK), but not jun N-terminal kinase (JNK), is necessary for convergent extension and initiation of mouse neurulation.

MATERIALS AND METHODS

Mouse strains and genotyping
CD1 random bred mice (Charles River, UK) and CBA/Ca inbred mice (Harlan Olac, UK) provided non-mutant embryos. Heterozygous males of the Lp/Le inbred strain, which carries the loop-tail (Lp) mutation (Jackson Labs, Bar Harbor, Maine) were mated with CBA/Ca females, and F1 Lp/+ offspring were intercrossed to generate embryos. Lp genotyping was as described (Stanier et al., 1995). The Crash (Crsh) mutation was originally detected in the F1 generation of ENU-treated strain 101/H males mated with C3H females. Crsh genotyping was by PCR amplification of flanking microsatellites D15Mit159 and D15Mit29 (Curtin et al., 2003). The Circletail (Crc) mutant arose as described (Rachel et al., 2000), with genotyping by PCR amplification of flanking microsatellites D15Mit144 and D15Mit68 (Murdoch et al., 2001b). Mice homozygous for the gene trap insertions cordon bleu (Cobl) and ROSA26 were as described (Gasca et al., 1995; Zambrowicz et al., 1997).

Embryo dissection and processing
Embryos were obtained from timed matings; noon on the day of finding a copulation plug was designated 0.5 days of embryonic development (E0.5). Pregnant females were killed by cervical dislocation and embryos were dissected from the uterus in Dulbecco’s Modified Eagle’s Medium (Gibco, UK) containing 10% fetal calf serum. Embryos for in situ hybridisation or histological analysis were washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight, or in Bouin’s fluid at room temperature for several days. Paraffin wax embedding was followed by sectioning at 8 μm and staining with Haematoxylin and Eosin (H&E). Embryos homozygous for the cordon bleu gene trap insertion were stained for bacterial β-galactosidase (β-gal) activity as described (Carroll et al., 2003).

Dil injection into the node of mouse embryos in vitro
Dil (1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, USA) was dissolved in 100% ethanol (0.5 mg/ml), then diluted tenfold in 3 mol/l sucrose. Node and midline injections were performed at E7.5 (allantoic bud stage) using a hand-held glass micropipette, while viewing the embryo from its posterior (node injection) or anterior (injections rostral to the node) surface, on the stage of a Zeiss SV6 stereomicroscope. The pipette tip was inserted into the node or midline region (Ybot-Gonzalez et al., 2002) and the Dil solution was released as the pipette was slowly withdrawn. Embryos were placed immediately into whole embryo culture.

Electroporation of intact mouse embryos
GFP-expressing constructs were pCAβ-mGFP6 and pCAβ-IRES-GFP (gifts of Dr Jonathan Gilthorpe), in which GFP expression is driven by the chicken β-actin promoter under influence of the cytomegalovirus enhancer (Yanera et al., 2002). Both constructs gave identical GFP-labelling results in embryo electroporation experiments. To prepare a vector expressing constitutively active RhoA, CDNA was amplified from vector pGEX-2T-RhoA-A63L (Self and Hall, 1995) using primers: 5’-GCATATCGATATGAGCTGC-

CATCAGGAAG-3’ and 5’-CAATGGCAGCCGTACACTACAGATGAGGCAC-3’. The amplified product was cloned into pGEM-T (Promega, UK) and then into pCAβ-mGFP6 to generate vector pCAβ-RhA-IRES-GFP, which was verified by DNA sequencing. Constitutively
active ROCK, pCAG-myc-P160 ROCK Δ3, wild-type ROCK, pCAG-myc-P160 ROCK, and an empty control vector, pCAG-myc, were as described previously (Gutjahr et al., 2005) (gifts of Dr Shuh Narumiya), and were co-electroporated with pCAβ2-BRES-GFP to enable identification of transfected cells. DNA solution for electroporation contained 4.5 mg/ml pCAβ2-H9252 or 2.5 mg/ml other vectors, plus 0.1% Fast Green. E7.5 embryos were placed in PBS in a Petri dish on a stereomicroscope stage and DNA solution was injected into the amniotic cavity using a hand-held glass micropipette. Embryos were immediately placed between a pair of gold 5 mm point electrodes (BTX model 508) attached to a BTX ECM830 electroporator, with five pulses, 50 milliseconds, 15 V) was passed, after which embryos were placed into culture.

**Chimera production and analysis**

Embryonic stem (ES) cells (129/SvEv; CCE cell line, a gift of Dr Elizabeth Robertson) were injected into E3.5 blastocysts produced from matings between Lp/+ mice that were also homozygous for the ROSA26 β-gal marker. Injected blastocysts were transferred to the uterus of E2.5 pseudopregnant recipients, with litters harvested at E8 to obtain chimeras during late gastrulation. After fixation in 4% paraformaldehyde, chimeras were immediately stained as whole-mounts by the X-Gal method (Carroll et al., 2003). Transverse sections were prepared following paraffin wax embedding, and cells of each genotype (β-gal-positive or β-gal-negative) were counted in defined areas of midline or lateral neural plate, with focussing at high magnification to distinguish the individual cells. Embryos were genotyped using DNA extracted from yolk sac endoderm, which is entirely host blastocyst-derived (Beddington and Robertson, 1989). Yolk sacs were incubated on ice for 1-5 hours in 2.5% pancreatin, 0.5% trypsin in Ca²⁺ or Mg²⁺-free PBS, after which the endodermal and mesodermal layers were peeled apart.

**Inhibitor treatment and analysis of embryos after culture**

Embryos were cultured (Copp et al., 1999) with addition of varying concentrations of Y27632 (688000; Calbiochem, UK) dissolved in sterile distilled water (0.5 to 2 μl of 5 mmol/l stock added per ml of rat serum) or SP600125 (420119; Calbiochem, UK) dissolved in dimethyl sulfoxide (0.5 to 2.5 μl of 20 mmol/l stock added per ml of rat serum). At the end of culture, extraembryonic membranes were removed and somites were counted (Copp et al., 1999). Embryos were inspected to determine whether neural tube closure had occurred at the site of Closure 1 (Copp et al., 1994).

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**Table 1. Frequency of rostrally directed extension of Dil- and GFP-labelled cells**

<table>
<thead>
<tr>
<th></th>
<th>No midline extension</th>
<th>Midline extension</th>
<th>Total</th>
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<tr>
<td><strong>1. Dil-injected embryos</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>2 (6)</td>
<td>33 (94)</td>
<td>35</td>
</tr>
<tr>
<td>Lp/+</td>
<td>12 (21)</td>
<td>46 (79)</td>
<td>58</td>
</tr>
<tr>
<td>Lp/Lp</td>
<td>18 (53)</td>
<td>16 (47)</td>
<td>34</td>
</tr>
</tbody>
</table>

Frequency of rostrally directed extension of labelled cells following injection of Dil or electroporation of a GFP expression vector into +/+, Lp/+ or Lp/Lp embryos at E7.5, followed by 18 hours development in whole-embryo culture (also see Fig. 1).

*Proportion of embryos undergoing midline extension differs significantly between genotypes (P<0.001) for both Dil and GFP labelling methods.

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**Fig. 2. Diminished length and increased width of Lp/Lp embryos.**

(A) Embryonic length and width as measured on photomicrographs of cultured embryos viewed from the dorsal surface, after removal of yolk sac and amnion. (B–E) Length and width measurements of embryos cultured in the absence (B, C) or presence (D, E) of the ROCK inhibitor Y27632. Each data point represents a single embryo. Linear regression lines are shown. Embryonic length increases significantly with somite number in both absence (B) and presence (D) of inhibitor (two-way ANOVA, P=0.001). Length also varies significantly with genotype (+/+ > Lp/Lp) in embryos cultured in the absence (B) of inhibitor (P=0.008), whereas in the presence of inhibitor (D) embryonic length no longer varies significantly with genotype (P=0.053). Embryonic width does not vary significantly with somite number either in the absence (C) or presence (E) of inhibitor (P>0.05). Whereas embryonic width varies significantly between genotypes (Lp/Lp > +/+) in the absence of inhibitor (P=0.006), there is no significant variation between genotypes in the presence of inhibitor (P>0.05). Embryonic width is significantly greater in the presence of inhibitor (E) than in its absence (C), irrespective of genotype (P<0.001), whereas embryonic length does not differ in the presence (D) or absence (B) of inhibitor (P>0.05). Number of embryos measured in absence or presence of Y27632 is: +/+: 35, 16; Lp/+: 56, 25; Lp/Lp: 28, 11.
DiI-injected or electroporated embryos were flat-mounted, dorsal side upwards in PBS on a glass microscope slide and photographed with a Leica DC 500 digital camera on a Leica MZ FL III stereomicroscope.

**In situ hybridisation analysis**

Whole-mount in situ hybridisation, followed by preparation of 50 μm vibratome sections, was performed using digoxygenn-labeled cRNA probes (Coppen et al., 1999; Ybot-Gonzalez et al., 2005). Previously published probes were: JNK1 (Kuan et al., 1999), Shh (Greene et al., 1998) and Vangl2 (Doudney et al., 2005). New cDNA probes were prepared by RT-PCR on total RNA from E10.5 CBA/Ca embryos using TRIZol reagent (Gibco BRL). Amplified fragments were cloned into the pGEM-T vector (Promega, UK) and sequenced to confirm identity. Primer sequences were: **ΔΔΔΔΔ**: forward 5'-TGACCTTGAGACAATGGGC-3' and reverse 5'-ACTCAGAGGTCTCATATGCC-3', amplifying a 416 bp fragment (nucleotides 330 to 745; GenBank NM_172464); **ΔΔΔΔ**: forward 5'-TTTTATAAGTGCACTCC-3' and reverse 5'-TTTGGTTTTGCTGATTTCC-3', amplifying a 501 bp fragment (nucleotides 11 to 511; GenBank AF178960); **ROCK1**: forward 5'-AGAACACCTTACAGTGGAGG-3' and reverse 5'-TATTGGACCTTGTCCG-3', amplifying a 412 bp fragment (nucleotides 1359 to 1770; GenBank U58513).

**RT-PCR analysis**

RNA was extracted from E7.5, 8.5 and 9.5 CD1 embryos using the PURESCRIPT RNA isolation kit (Gentra Systems, UK). DNA was removed with a DNA-free kit (Ambion, UK), and cDNA was synthesised using a Superscript First Strand Synthesis System (Invitrogen, UK). PCR products were analysed on ethidium bromide-stained 1% agarose gels using Hyperladder I (Bioline, UK) as size markers. Primers were: **ΔΔΔΔΔ**: forward 5'-TACCTTGAGAACATGGACACC-3' and reverse 5'-CCCTGAGATTTGCC-3', amplifying a 447 bp fragment (nucleotides 2295 to 2741; GenBank NM_010717); **ΔΔΔΔΔ**: forward 5'-CTTGTTGATTGAGGTAAGTCGG-3' and reverse 5'-TTTAGACCCCAAAGTCTGAGC-3', amplifying a 510 bp fragment (nucleotides 2330 to 2839; GenBank AB008117); **ΔΔΔΔΔ**: forward 5'-CAAGCTTGGAGACGATACCC-3' and reverse 5'-CTTGCTGCTTGTGACTTGG-3', amplifying a 505 bp fragment (nucleotides 1299 to 1803; GenBank U58512).

**Cell culture and immunoblotting**

293T cells were cultured by standard methods, plasmids were introduced by calcium phosphate transfection, and cells were lysed in 50 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 1% Triton X100 containing protease inhibitors (Complete; Roche). Protein assays were performed on cell extracts and 7.5, 150 mmol/l NaCl, 1% Triton X100 containing calcium phosphate transfection, and cells were lysed in 50 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 1% Triton X100 containing protease inhibitors (Complete; Roche). Protein assays were performed on cell extracts and samples containing 10 μg total protein were electrophoresed. Immunoblots were performed as described previously (Greene et al., 2002), using an antibody for phosphofurin (1 in 1000 dilution; Cell Signalling Technology). Blots were stripped and re-probed with an antibody to β-tubulin (1 in 3000 dilution; Santa Cruz Biotechnology Inc.).

**Statistical analysis**

Length and width data were subjected to two-way analysis of variance, with Lp genotype and somite number as variables. Response to ROCK and JNK inhibitors (proportion of embryos with open neural tube) was compared between genotypes by z-test. All statistics were computed using SigmaStat v.2 (SPSS Inc.).

**RESULTS**

**Defective convergent extension in Lp (Vangl2) mutant mouse embryos**

To determine whether axial extension is compromised in the loop-tail (Vangl2) mouse mutant, before failure of initiation of neural tube closure (Closure 1), we labelled the extending midline by focal injections of DiI into the node at E7.5. Embryos of all genotypes were labelled with equal intensity, which was performed blind to embryo genotype (see Fig. 1A in the supplementary material). Following 18 hours development in whole embryo culture, rostrally

**Fig. 3. Cell-autonomous defect of convergent extension in the Lp mutant as demonstrated in chimeras. (A-C) +/- ↔ +/- and (D-F) +/- ↔ Lp/Lp chimeras at E8, produced by injecting wild-type ES cells into Lp blastocysts that are also homozygous for the ROSA26 β-gal marker. After X-Gal staining and Eosin counterstaining, all blastocyst-derived cells are blue, while ES-derived cells are pink. Sections at the level of the notochordal plate (A,D), mid-trunk neural folds (B,E) and cranial neural folds (C,F) all show an apparently random admixture of blue and pink cells in +/- ↔ +/- chimeras whereas +/- ↔ Lp/Lp chimeras (D-F) exhibit increased numbers of pink (wild-type) cells in the midline of both neural plate (arrows) and notochordal plate (asterisk in D). More lateral regions show a more even mix of blue and pink cells. Inset: low magnification view of +/- ↔ Lp/Lp chimera showing boxed areas in which cell counts were made. (G) Quantitative analysis of relative cell numbers in midline and lateral neural plate regions of different chimera genotypes. Difference in % β-gal-negative cell content in midline and lateral neural plate regions of different chimera genotypes. Difference in % β-gal-negative (i.e. wild-type) cells between midline and lateral regions gives values close to zero at all axial levels of +/- ↔ +/- chimeras whereas +/- ↔ Lp/Lp chimeras show values deviating markedly from zero along the entire body axis. +/- ↔ Lp/Lp chimeras show a wide range of values, encompassing the extremes of the other two genotypes. Scale bar: in A, 0.05 mm for A-F.**
Convergent extension and neurulation

Directed extension of DiI-labelled cells was observed in 94% of wild-type embryos (Fig. 1A; Table 1). By contrast, only 47% of Lp/Lp embryos exhibited rostral extension, with labelled cells persisting at the site of node injection in the remaining 53% of homozygotes (Fig. 1C). Among Lp heterozygotes, 79% showed some rostral extension, but with considerable variability between embryos (Fig. 1B). Embryo sections demonstrate that node injection labels predominantly midline axial mesoderm in the caudal region (Fig. 1G), the notochord along the more rostral body axis (Fig. 1H,I), and midline caudal neural plate in some embryos (Fig. 1G).

As an alternative approach, we labelled midline neural plate cells in the caudal region of E7.5 embryos by electroporation from the amniotic cavity of a DNA vector expressing green fluorescent protein (GFP) under control of the β-actin promoter. GFP expression could be detected from 4 hours post-electroporation (see Fig. S1B in the supplementary material). Following 18 hours development in whole embryo culture, we observed marked midline extension of GFP-expressing cells in all wild-type (Fig. 1D) and in most Lp/+ embryos (Fig. 1E), whereas all Lp/Lp embryos exhibited greatly reduced midline extension (Fig. 1F; Table 1). Staining embryo sections with an anti-GFP antibody confirmed that labelling was in the neural plate (Fig. 1J). These findings demonstrate a defect of convergent extension, affecting both axial mesoderm and neural plate, in pre-neurulation stage homozygous loop-tail mutant embryos.

Length and width measurements of DiI-labelled embryos following the culture period also support a defect in convergent extension (Fig. 2A-C). Embryonic length was significantly greater in somite-matched +/- embryos compared with Lp/Lp littermates, whereas embryonic width showed the reverse trend: Lp/Lp embryos were significantly wider than +/- littermates. Lp/+ embryos gave an intermediate result in both cases.

Cell-autonomous defect of convergent extension

In Drosophila, epithelial PCP effects can be cell autonomous, as in the distal positioning of wing hairs, or non-cell autonomous, as in their long-range polarisation (Strutt, 2003). To investigate the cell autonomy of defective convergent extension in the Lp mutant, we constructed chimeras by injecting wild-type ES cells into blastocysts from Lp/+ × Lp/+ matings. Host blastocysts were also homozygous for the ROSA26 β-gal marker, enabling cells of the two genotypes within the chimera to be distinguished in tissue sections. Cells originating from the host blastocyst (β-gal-positive) and from the donor ES cells (β-gal-negative) exhibited an apparently random, fine-grained admixture in both the midline and lateral neural plate.
of E8 chimeras with +/+ vs. +/+ genotype (Fig. 3A-C). By contrast, the midline neural plate of +/+ vs. Lp/Lp chimeras appeared to contain a lower proportion of β-gal-positive (Lp/Lp) cells than did lateral regions (Fig. 3D-F). Similarly, we observed a predominance of β-gal-negative cells in the node-derived notochordal plate of +/+ vs. Lp/Lp chimeras (Fig. 3D) whereas an even mixture of β-gal-positive and -negative cells was apparent in the notochordal plate of +/+ genotype (Fig. 3A).

To investigate in more detail this apparent genotype-specific difference in colonisation of the midline, we counted the number of β-gal-positive and -negative cells in standardised areas of midline and lateral neural plate at three axial levels in chimeras of +/+ vs. Lp/Lp genotypes (n=3 embryos per genotype). Individual chimeras are expected to differ from each other in their overall proportion of ES-cell-derived and blastocyst-derived components. To eliminate this variation, we subtracted the per cent of β-gal-negative cells in the lateral region from that in the midline of each chimera. Hence, a chimera with +/+ vs. +/+ chimeras did indeed yield values clustered around zero (Fig. 3G).

By contrast, +/+ vs. Lp/Lp chimera sections showed a 15-45% predominance of wild-type cells over Lp/Lp cells in the midline compared with the lateral neural plate. This effect was present similarly in caudal, middle and cranial regions of the chimeras. Interestingly, a wide variation of values was detected in +/+ vs. Lp/Lp chimeras. Some sections showed a midline-lateral difference close to zero, whereas others showed values similar to those found in +/+ vs. Lp/Lp chimeras. We conclude that the colonisation of the midline by Lp/Lp cells is intrinsically defective and is not rescued by close juxtaposition to wild-type cells in a chimeric combination.

**Mechanism underlying defective midline extension in Lp**

Injection of DiI into the wild-type mouse node at E7.5 labels midline chordamesodermal cells along the whole body axis (Fig. 1A) (Beddington, 1994; Sulik et al., 1994). This is consistent with a model derived from avian studies in which Hensen’s node ‘regresses’ during gastrulation, laying down the entire notochord (Catala et al., 1996). In mice, a notochord is lacking from embryos following surgical or genetic ablation of the node (Davidson et al., 1999; Ang and Rossant, 1994; Weinstein et al., 1994; Ybot-Gonzalez et al., 2002). Hence, we considered the possibility that the defect of axial extension in Lp/Lp embryos may result from a defect in the node. To examine node morphology and gene expression, we bred the cordon bleu (Cobl) gene trap, which expresses β-gal specifically in the node and its derivatives (Carroll et al., 2003), onto the Lp strain. At E7.5, Lp/Lp embryos exhibited a β-gal-positive node that was morphologically identical to the wild-type node, with closely similar cell numbers (Fig. 4A-D). By contrast, at E8.5, we found a markedly broader β-gal-positive node in Lp/Lp embryos than in wild type (Fig. 4E,F). The expression domain of sonic hedgehog (Shh) was also broader in the notochord, and particularly in the node and notochordal plate, of Lp/Lp embryos compared with +/+ littermates (Fig. 4G-I). We previously also found a broader expression domain of brachyury, in the Lp/Lp primitive streak and node (Greene et al., 1998). These findings suggest a defect of midline cell intercalation in Lp, particularly affecting the node and cells recently emerged from the node.

To determine whether notochordal extension at more rostral levels is also affected in Lp embryos, we injected DiI at midline positions rostral to the E7.5 node (Fig. 4J-L). Rostral injections label a stream of midline notochordal cells extending variable distances along the body axis, often into the cranial region, but not...
including the node itself (Fig. 4M-O). In Lp/Lp embryos, we observed a similar range of labelling patterns as in wild-type and Lp/+ embryos (Fig. 4P), demonstrating that notochordal extension rostral to the node and notochordal plate are not markedly abnormal in the Lp mutant.

**Requirement for RhoA-ROCK signalling**

We asked whether mouse convergent extension and initiation of neural tube closure require specific signalling downstream of the PCP genes. Signalling via RhoA-ROCK has been identified in *Drosophila*, *Xenopus* and zebrafish (Strutt et al., 1997; Habas et al., 2001; Marlow et al., 2002), and via JNK in *Drosophila* and *Xenopus* (Boutros et al., 1998; Yamanaka et al., 2002). Moreover, blockade of ROCK function causes defective neural tube closure in the chick (Wei et al., 2001). RT-PCR shows expression of RhoA, ROCK1 and ROCK2 from E7.5 to 9.5 of mouse development, together with targets of ROCK phosphorylation including LIM kinase (LIMK)-1 and -2 (Maekawa et al., 1999) (Fig. 5A). In situ hybridisation at late gastrulation (E8), when convergent extension is underway, revealed expression of the PCP pathway components Vangl2, Daam1, RhoA and ROCK2 throughout the embryo (Fig. 5B-E), whereas at E8.5, expression of Vangl2 and Daam1 became restricted to the region of hindbrain and upper spine, where neural tube closure is initiated (Fig. 5F,G). At this stage, RhoA, ROCK2 and JNK1 were expressed in domains that overlap with the PCP genes (compare Fig. 5H-J with F,G). Transcripts are present in both neural tube and axial and paraxial mesoderm (not shown).

To examine the functional requirement for RhoA and ROCK, or JNK signalling in mouse convergent extension and closure initiation, we cultured E7.5 +/+ and Lp/+ embryos for 18 hours in varying concentrations of the ROCK inhibitor Y27632. Inhibition of ROCK strongly summated with genotype at the Lp locus, with Lp heterozygotes exhibiting almost 100% open neural tubes even at the lowest concentration of Y27632 tested (2.5 μmol/l). By contrast, 2.5-5 μmol/l inhibitor had no adverse effect on neural tube closure in +/+ embryos, with non-closure first observed in 50% of embryos only at the near-toxic dose of 10 μmol/l (Fig. 6A; Table 2). We also identified a hallmark of ROCK-dependent failure of Closure 1 resembled that seen in Lp/Lp, and seems unlikely to be a non-specific toxic effect.

By contrast to the findings with Y27632, inhibition of JNK activity by SP600125 did not summate with the Lp mutation. Both +/+ and Lp/+ embryos completed Closure 1 in the presence of inhibitor (Fig. 6B), even at a near-toxic concentration (50 μmol/l) far in excess of that typically used in cell cultures. This result is consistent with the successful Closure 1 seen in mouse embryos lacking function of both JNK-1 and -2 (Kuan et al., 1999).

The PCP mutants Crash and Crc also showed an interaction with ROCK inhibition. Crash/+ embryos had almost 100% open neural tubes at 2.5 μmol/l Y27632, a concentration that had no effect on +/+ littermates (Fig. 6C). Crc/+ embryos were more resistant: open neural tubes first occurred at 5 μmol/l Y27632 and, even at 10 μmol/l, only 67% of heterozygotes failed to complete Closure 1 (Fig. 6D). This relative resistance of Crc/+ embryos to Y27632 may be an effect of genetic background, as wild-type littermates were also resistant to Y27632. Alternatively, the molecular pathway downstream of Srb1 may differ subtly from that of the proven PCP genes Vangl2 and Celsr1.

**Table 2. Frequency of open neural tubes (failure of Closure 1) among wild-type and heterozygous embryos of the Lp, Crash and Crc mutant strains treated with Y27632 and SP600125**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>Wild type</th>
<th>Heterozygote</th>
<th>Z-test</th>
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*Significant difference between proportion of open neural tubes in wild-type and heterozygous embryos by Z-test, P<0.05.
Length and width measurements in \(Lp\) embryos treated with Y27632 (Fig. 2D,E) revealed a persistent effect of \(Lp\) genotype similar to that of embryos cultured without inhibitor. While Y27632 did not affect embryonic length after culture, embryonic width was significantly greater in all genotypes, suggesting that inhibition of RhoA and ROCK signalling may particularly diminish lateral-to-medial cell movements during gastrulation.

**Transfection of active RhoA and ROCK into \(Lp\) embryos**

We attempted to rescue *loop-tail* mutants from CRN by electroporating the midline neural plate with DNA constructs expressing constitutively active forms of RhoA or ROCK. Unexpectedly, both treatments exacerbated the mutant effect, rather than ameliorating it, so that the majority of electroporated \(Lp/\pm\) embryos, and even a proportion of \(+/\pm\) embryos, failed in Closure 1 like \(Lp/Lp\) littermates (Table 3). Transfected GFP-positive embryonic cells were sparse and clumped (Fig. 8B,C,E,F) compared with cells electroporated with wild-type ROCK (Fig. 8D), or an empty control vector (Fig. 8A), raising the possibility that some cells transfected with active RhoA or ROCK may have died. In vitro, 293T cells transfected with active RhoA survived but were rounded, with reduced cellular protrusions (Fig. 8G,H), while immunoblotting extracts of 293T cells transfected with active RhoA showed diminished abundance of phosphocofilin, a downstream phosphorylation target of the RhoA-ROCK pathway (Fig. 8I). Hence, transfection of constitutively active RhoA or ROCK appears to inhibit downstream signalling, consistent with recent findings (Gutjahr et al., 2005), and providing a likely explanation for the detrimental effect of transfection on neural tube closure.

**DISCUSSION**

During gastrulation in mammals, two convergent extension processes drive the narrowing and lengthening of the body axis that typifies embryos in the immediate pre-neurulation period. As summarised in Fig. 9, chordamesodermal cells emerge from the node and intercalate in the midline, as the notochordal plate narrows and intercites in the midline, with a few positive floor plate cells (white arrow in K). In the inhibitor-treated embryo (I; higher magnification in I), Shh expression is similarly in the notochord but also in a laterally expanded floor plate domain (fp). Scale bars: in F, 0.4 mm for A,B,E,F; in C, 0.1 mm for C,D; in G, 0.05 mm for G,H; in I, 0.1 mm for I,J; in K, 0.025 mm for K,L.
Dil injection into the node identifies reduced extension in midline mesoderm, whereas more rostral labelling of the notochord does not reveal differences in extension behaviour between Lp mutant and wild-type embryos. This suggests that, although convergent extension is a key factor in the narrowing and elongation of the notochordal plate immediately in front of the node, elongation of the notochord more rostrally is less dependent on convergent extension. Indeed, morphometric analysis of chick and mouse gastrulation shows that cell rearrangement is a relatively minor contributor to notochord elongation at levels rostral to the notochordal plate (Sausedo and Schoenwolf, 1993; Sausedo and Schoenwolf, 1994). Longitudinally oriented mitoses appear to account for the majority of the elongation observed in the rostral notochord. We suggest that unimpeded extension of the notochord is the primary factor enabling the limited degree of axial extension that is achieved by Lp/Lp embryos.

Defective convergent extension in the Lp neural plate could indicate an intrinsic requirement for Vangl2 function, or a secondary effect of faulty induction by the chordamesoderm. The notochord induces not only expression of floor plate markers such as Shh and netrin-1 in the midline neural plate (Jessell, 2000), but also influences earlier cell morphogenesis. For example, neuroepithelial cells adopt a wedge shape during formation of the median hinge point (MHP) in response to notochordal influence (Smith and Schoenwolf, 1989). Hence, faulty midline cell intercalation in the Lp/Lp neural plate could reflect a disturbance of notochordal induction. However, Vangl2 and other PCP genes are expressed in the early neural plate (Kibar et al., 2001; Murdoch et al., 2001a; Torban et al., 2007), consistent with a direct requirement for PCP signalling during neurcetodermal convergent extension. Moreover, our chimera studies suggest that Lp/Lp neural plate cells are preferentially affected in convergent extension, compared with wild-type cells. If the neural plate defect was secondary to faulty notochordal induction, both wild-type and mutant neural plate cells should be equally affected. It seems likely, therefore, that convergent extension requires PCP signalling independently in each germ layer.

The absence of Lp mutant cells from the midline of chimeras could reflect a role for Vangl2 in cell behaviours other than convergent extension. For example, differential cell adhesion may underlie the morphogenetic disturbance in PCP mutant zebrafish (Ciruna et al., 2006). In mouse chimeras, however, differentially adhesive cells tend to show marked spatial segregation (Tam and Rossant, 2003), a phenomenon that we did not observe in +/+ → Lp/Lp chimeras. It is also possible that the differential abundance of Lp mutant and wild-type cells in the midline reflects proliferative differences, as in zebrafish PCP mutants in which mitotic orientation is disturbed (Gong et al., 2004). However, we previously observed no differences in cell proliferation between Lp/Lp and wild-type neural plate cells (Gerrelli and Copp, 1997). In the absence of other likely explanations, therefore, the predominance of wild-type cells in the midline of +/+ → Lp/Lp chimeras supports a cell-autonomous defect of convergent extension.

PCP signalling via small GTPases regulates intracellular events such as cytoskeletal remodelling that underlie polarised cell behaviour (Klein and Mlodzik, 2005). The apparently cell-autonomous effect on convergent extension in Lp, with a requirement for RhoA-ROCK signalling, is consistent with this model. However, both cell-autonomous and non-cell-autonomous

### Table 3. Frequency of open neural tubes (failure of Closure 1) among +/+, Lp/+ and Lp/Lp embryos electroporated with vectors expressing constitutively active RhoA or ROCK, or wild-type ROCK

<table>
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<tr>
<th>Construct</th>
<th>No. embryos with open neural tube/Total no. embryos</th>
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<tr>
<td>Empty vector</td>
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<tr>
<td>RhoA active</td>
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<tr>
<td>ROCK active</td>
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</tr>
<tr>
<td>ROCK wild type</td>
<td>1/4 2/9 1/1</td>
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### Fig. 8. Electroporation of constitutively active RhoA or ROCK, or wild-type ROCK, disrupts convergent extension in Lp/+ and +/+ embryos. (A-F) Lp/+ and +/+ embryos after 18 hours culture following electroporation in the node region with vector only (A), RhoA active (B,E) or ROCK active (C,F) or ROCK wild type (D). The vector control shows marked midline extension of GFP-positive cells (arrows in A), by contrast to the sparse, clumped appearance of cells electroporated with RhoA active (arrows in E) or ROCK active (arrows in C,F). Electroporation with ROCK wild type allows greater midline extension (arrows in D) although less than in the vector control. (G,I) 293T cells transfected with RhoA active (H) show reduced protrusive behaviour compared with vector-only controls (G). (I) Immunoblot for phospho-cofilin in 293T cells transfected with vector only (lane 1), ROCK active (lane 2), ROCK wild type (lane 3) or ROCK active (lane 4). Beta tubulin (lower panel) serves as a loading control. Note the severely reduced phosphocofilin abundance in cells expressing constitutively active RhoA and mild downregulation of phosphocofilin in cells expressing wild type and constitutively active ROCK. Scale bar: in F, 0.4 mm for A-F.
Fig. 9. Summary of the convergent extension defect in Lp. Mesodermal cells emerge from the node into the notochordal plate, where midline intercalation leads to narrowing to form the notochord. In the overlying neural plate, cells converge on the midline, where they extend in the rostrocaudal axis. Red arrows: events that are disrupted in Lp/Lp embryos: principally midline intercalation in cells emerging from the node and convergent extension in the neural plate. Black arrows: events that appear to occur normally in Lp/Lp embryos.

features of PCP signalling have been described in other systems. For example, whereas mediolateral cell alignment during zebrafish convergent extension requires both Vangl2 (Stbm), and Rho kinase 2 (Rok2), wild-type cells also fail to align when transplanted to hosts deficient in Stbm or Rok2 (Marlow et al., 2002; Jessen et al., 2002). Conversely, overexpression of Xenopus Vangl2 (Xstbm) prevents cell intercalation, but can be rescued if overexpressing cells are juxtaposed to wild-type cells (Goto and Keller, 2002). In both Xenopus and Drosophila, non-cell-autonomous effects of PCP signalling are generally short-range, often extending a few cell diameters from the edge of single-genotype patches in mosaics (Goto and Keller, 2002; Strutt, 2003). By contrast, mutant and wild-type cells were closely intermingled in our mouse chimeras, without distinct patches of either genotype. Hence, the conditions necessary to demonstrate non-cell-autonomous effects may have been absent, so that only cell-autonomous, single-cell effects were evident.

Neural tube defects are common human malformations, occurring on average in 1 per 1000 pregnancies worldwide. Cranial defects are lethal, and survivors with spinal malformations often exhibit considerable disability, starting in childhood and extending into adult life (Botto et al., 1999). Despite their clinical importance, the genetic predisposition to human neural tube defects is poorly understood. The finding that folic acid can prevent a proportion of neural tube defects in the mammalian neurulation (Boyles et al., 2005). Among the PCP genes, sequence variants of VANGL2 and its family member VANGL1 were not found to be associated with neural tube defects (Doudney et al., 2005). The present study suggests that other PCP genes should be considered as candidates for a role in the aetiology of human neural tube defects.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/4/789/DC1

References


