Targeted deletion of the novel cytoplasmic dynein mD2LIC disrupts the embryonic organiser, formation of the body axes and specification of ventral cell fates

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Accepted 23 July 2004
Published by The Company of Biologists 2004
doi:10.1242/dev.01389

Summary

Dyneins have been implicated in left-right axis determination during embryonic development and in a variety of human genetic syndromes. In this paper, we study the recently discovered mouse dynein 2 light intermediate chain (mD2LIC), which is believed to be involved in retrograde intraflagella transport and which, like left-right dynein, is expressed in the node of the mouse embryo. Cells of the ventral node of mouse embryos lacking mD2LIC have an altered morphology and lack monocilia, and expression of Foxa2 and Shh in this structure is reduced or completely absent. At later stages, consistent with the absence of nodal cilia, mD2LIC is required for the establishment of the left-right axis and for normal expression of Nodal, and the ventral neural tube fails to express Shh, Foxa2 and Ebaf. mD2LIC also functions indirectly in the survival of anterior definitive endoderm and in the maintenance of the anterior neural ridge, probably through maintenance of Foxa2/Hnf3β expression. Together, our results indicate that mD2LIC is required to maintain or establish ventral cell fates and for correct signalling by the organiser and midline, and they identify the first embryonic function of a vertebrate cytoplasmic dynein.

Key words: D2LIC, Axis formation, Handedness, Mouse embryo, Intra-flagellar transport, Lefty2, Hnf3β

Introduction

Dynein is a large multi-subunit protein complex consisting of two heavy chains complexed to intermediate, light intermediate and/or light chains (Harrison and King, 2000). The dynein family has been divided into two subfamilies: the axonemal and cytoplasmic dyneins. Axonemal dyneins function as structural elements in cilia and flagella, and provide the force responsible for ciliary motion. By contrast, cytoplasmic dyneins are motor proteins involved in the intracellular transport of cargo along microtubules and in retrograde intraflagellar transport during the assembly of cilia (Perrone et al., 2003).

The dyneins have been implicated in many cellular and developmental processes. For example, the gene responsible for the spontaneous classical mouse mutation inversus viscerum (iv) is an axonemal heavy chain dynein named left-right dynein (previously lrd; Dnahc11 – Mouse Genome Informatics) (Supp et al., 1997). Dnahc11 is expressed in the node of the mouse embryo and is necessary for the motility of cilia in the node. It is required for the establishment of the left-right body axis and has been implicated in Kartagener’s and other immotile cilia syndromes (Supp et al., 1997). In addition, dyneins have been associated with severe clinical abnormalities, such as heterotaxia and isomerism (including polysplenia or asplenia), and with single organ inversions, such as dextrocardia (Casey and Hackett, 2000), as well as in human genetic disease syndromes including spinal bulbar muscular atrophy and spinal muscular atrophy (Hafezparast et al., 2003).

The importance of intra-flagellar transport in embryonic development has been emphasised by analysis of wimple (previously Wim; Ift172 – Mouse Genome Informatics) and flexo (previously Fxo; Tg737Rpw – Mouse Genome Informatics) (Huangfu et al., 2003), which, together with the kinesin Kif3a (Marszalek et al., 1999; Takeda et al., 1999), are involved in anterograde intra-flagellar transport. Mouse mutants lacking Wim or Fxo fail to specify ventral cell fates, probably because Shh signalling is disrupted downstream of the patched receptor (Huangfu et al., 2003).

Together, these observations emphasise that the study of dyneins is of great cellular, developmental and medical interest. Recently, on the basis of its amino acid sequence and
its ability to interact with cytoplasmic dynein 2 heavy chain (DHC2). Grissom and colleagues (Grissom et al., 2002) have classified dynein 2 light intermediate chain (D2LIC) as a novel member of the dynein family of proteins. Consistent with this proposal, XBX-1, the Caenorhabditis elegans homologue of D2LIC, proves to be required for retrograde intraflagellar transport (Schafer et al., 2003). We previously identified mouse D2LIC (mD2LIC; 4933404011Rik – Mouse Genome Informatics) as a gene that is expressed in the node of the developing embryo (Sousa-Nunes et al., 2003), and in this paper we investigate its function during development. Our work shows that mD2LIC is needed to maintain or establish ventral cell fates, for monocilium formation in the ventral node, and for correct signalling by the organiser and midline. Our experiments define the first embryonic function for a vertebrate cytoplasmic dynein.

**Materials and methods**

**In situ hybridisation**

In situ hybridisation of mD2LIC was performed as described (Wilkinson and Nieto, 1993) using probes derived from EST clone AL024282. Probes specific for other genes are described in the text. The lefty probe (Meno et al., 1997) detects both Left1 (previously lefty1) and Ebaf (previously lefty2) transcripts.

**Northern blot analysis**

Total RNA was isolated from adult tissues and from 11.5 dpc whole embryos and placenta as described (Chomczynski and Sacchi, 1987). A probe was prepared from a full-length mD2LIC cDNA. Hybridisation was performed as described (Martinez-Barbera et al., 1997).

**Scanning electron microscopy**

Scanning electron microscopy was performed as described (Sulik et al., 1994), with 15 minutes uranyl acetate treatment.

**TUNEL staining**

TUNEL staining was performed as described (Barbera et al., 2002).

**Gene targeting**

Genomic clones were isolated from a 129/OlaC genomic library (Stratagene) using an mD2LIC full-length cDNA to generate a probe. A 2.75 kb region of the endogenous genomic locus was replaced by homologous recombination in E14TG2A ES cells with a 2 kb loxp flanked PGK neomycin (PGKneo) cassette using a diphtheria toxin A (D7A) cassette for negative selection (Fig. 3). Correctly targeted cells from five independent cell lines were used to generate chimeric animals that were subsequently mated to produce heterozygous individuals. Only heterozygotes derived from the same cell line were intercrossed. The phenotypes of homozygous individuals derived from each of the five cell lines were similar, indicating that the embryonic defects observed in this paper are consequences of the targeting event.

**Genotyping of wild-type and mutant mD2LIC alleles**

Individuals were genotyped using the polymerase chain reaction (PCR) or by Southern blot analysis. In the PCR strategy, a 450 bp product specific to the wild-type locus was amplified using the primers P1 (5’-GCCCAACATTGTCTCAGCTTC-3’) and P2 (5’-TGACA-GCGAGGTACTACTGCT-3’), and a 350 bp neomycin-specific PCR product was amplified using the primers 5’-CAAG-ATGAATCGAGGCCG-3’ and 5’-CGCGAAGCAGAGGCTGA-GAT-3’.Southern blotting was performed as described using a 700 bp probe that lies externally to the targeted region and recognises 13 kb (wild-type locus) and 6 kb (targeted locus) genomic fragments following digestion with HindIII (see Fig. 3).

**Results**

**Sequence and expression pattern of mD2LIC**

In contrast to Grissom and colleagues (Grissom et al., 2002), we find that mD2LIC protein is predicted to contain 351 amino acids, with a putative P-loop (nucleotide binding domain) near its N terminus (Fig. 1). We believe that this discrepancy has arisen because Grissom and colleagues (Grissom et al., 2002) made use of an EST sequence (mouse locus AK008822, BAB25915; M. musculus Genome Sequencing Consortium) that contains a frame shift relative to the sequence presented in our study; when translated, this results in a 209 amino acid protein that lacks the P-loop domain. Our translation of the mD2LIC cDNA (Sousa-Nunes et al., 2003) yields a protein that closely resembles the 350 amino acid human D2LIC, such that there is 87% identity and 92% amino acid charge matches between the two proteins.

Expression of mD2LIC in the late streak to early somite stage mouse embryo is restricted to the monociliated cells of the ventral node (Sousa-Nunes et al., 2003) (Fig. 2A-E). Northern blot analysis reveals a single transcript of 1.4 kb, and in the adult strongest expression of mD2LIC occurs in kidney and brain (Fig. 2F). The initial expression pattern of mD2LIC resembles that of the axonemal left-right dynein (lrd), suggesting that mD2LIC, like lrd, is involved in establishment of the embryonic axis (Supp et al., 1999).

**Targeted mutation of mD2LIC**

To analyse the function of mD2LIC, we generated a targeted mutation in embryonic stem cells. The mutation results in loss of both transcriptional and translational start sites and is likely to represent a null allele (Fig. 3A-D). mD2LIC−/− mutants die before 11.5 days post coitum (dpc) (Table 1) and gross phenotypic effects are detectable from 8.5 dpc, with defects in notochord and floorplate formation and a reduction in definitive endoderm. These are followed by anterior truncations of the forebrain, defects in the ventral body wall and in closure of the neural tube, and either an arrest of embryonic turning and heart looping or a randomisation in their direction (Fig. 3E; Table 2). The severity of this phenotype varies from embryo to embryo, and we have defined three phenotypic classes (Fig. 3E; Table 2). Class I, the most severe, was observed most frequently, and our analysis therefore concentrates on such embryos unless stated otherwise.

The gross phenotype at 8.5 dpc and later is presaged at 7.5 dpc by a disruption of cilium formation in the ventral node, where expression of mD2LIC is highest. Although scanning electron microscopy revealed that the gross morphology of the node is normal in null mutants (Fig. 4A,C,E), the single cilium that is normally carried by ventral node cells was absent in four out of 12 null mutant embryos. A further eight embryos formed only stunted cilium-like structures that were restricted to the posterior region of the node (Fig. 4B,D,F-I). Cells of the notochordal plate lacked cilia completely, suggesting that the stunted cilium-like structures visible in the node of some embryos are later lost, perhaps through reabsorption. We note that cells in the node and notochordal
plate also appear flatter than their wild-type counterparts (Fig. 4B,D,F-I).

**Gene expression patterns in mD2LIC–/– mutant embryos**

The abnormal appearance of the node suggests that these cells might be incorrectly specified. To address this point, we examined the expression patterns of *Fgf8, Shh, T* and *Foxa2* (previously Hnf3b) (Ang and Rossant, 1994; Crossley and Martin, 1995; Echelard et al., 1993; Wilkinson et al., 1990). At the late streak stage, expression of *Fgf8* is normal in null mutant embryos (Fig. 5A,B), while expression of both *Shh* and *T* is slightly reduced. Most significantly, expression of Foxa2 in the region of the node was severely reduced in mD2LIC–/– embryos (n=7/9), and in some was almost undetectable (n=2/9) (Fig. 5F-J).

Gene expression patterns in derivatives of the node are also disrupted. *T* (Fig. 6A,B) and *Shh* (data not shown) are both expressed in a discontinuous manner in the region of the notochord, while *Foxa2* is completely absent here and in the plate of null mutant embryos, and its expression in foregut endoderm is markedly reduced (Fig. 6E,F). *Shh* transcripts are also absent from the floor plate and endoderm of mD2LIC–/– embryos (Fig. 6C,D).

The downregulation of these genes is unlikely to be due to cell death; TUNEL staining revealed no abnormal levels of apoptosis in the node or its derivatives at any of the stages examined (Fig. 5K-M). Consistent with the downregulation of *T, Shh* and Foxa2 in the midline of mD2LIC–/– embryos, histological analysis revealed that loss of mD2LIC impairs differentiation of the notochord (see Table 3).

**Disruption of signalling by the node and axial mesoderm in mD2LIC–/– mutant embryos**

The signalling properties of the node and axial mesendoderm in mD2LIC–/– mutant embryos (n=5/5; Fig. 5C-E) and *T* (n=5/5; data not shown) is slightly reduced. Most significantly, expression of Foxa2 in the region of the node was severely reduced in mD2LIC–/– embryos (n=7/9), and in some was almost undetectable (n=2/9) (Fig. SF-J).
in mD2LIC−/− mutant embryos, and the establishment of the left-right axis, were further investigated by examining the expression of Nodal, Leftb, Ebaf, and Pitx2 (Collignon et al., 1996; Kitamura et al., 1999; Meno et al., 1997). Nodal is the earliest known asymmetric marker of the left-right axis. In wild-type embryos expression first occurs symmetrically around the periphery of the node, but it then becomes asymmetrical, with higher levels and a broader domain of expression on the left-hand side. nodal is then also expressed in the left-hand lateral plate. At late streak stages nodal expression was reduced in three mD2LIC−/− embryos, was absent in one, and, in contrast to its normal expression pattern, was expressed symmetrically around the node in eight (Fig. 6G,H and data not shown). At later stages (2-5 somite stages), left-sided (n=2/9), right-sided (n=1/9) and bilateral (n=5/9) expression patterns were observed in the lateral plate, and in one embryo (n=1/9) no expression was detectable. We note that the frequency of bilateral expression is similar to the frequency of the more severe Class I and II mutants (Table 2). Expression of Leftb was undetectable in all null embryos (n=5/5), while Ebaf expression occurred bilaterally in three out of five embryos, was expressed on the right-hand side in another, and was absent in a fifth (Fig. 6L,M). Similar results were observed with Pitx2 (Fig. 6N-Q). Together, these results indicate that

### Table 1. Frequency of individuals recovered from heterozygous intercrosses

<table>
<thead>
<tr>
<th>Stage (dpc)</th>
<th>Number of embryos</th>
<th>+/+ (%)</th>
<th>+/− (%)</th>
<th>−/− (%)</th>
</tr>
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<tbody>
<tr>
<td>7.5</td>
<td>317</td>
<td>29</td>
<td>48</td>
<td>23</td>
</tr>
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<td>8.5</td>
<td>198</td>
<td>23</td>
<td>50</td>
<td>27</td>
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<td>9.5</td>
<td>169</td>
<td>33</td>
<td>44</td>
<td>23</td>
</tr>
<tr>
<td>10.5</td>
<td>32</td>
<td>28</td>
<td>53</td>
<td>19</td>
</tr>
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<td>11.5</td>
<td>12</td>
<td>34</td>
<td>58</td>
<td>8</td>
</tr>
<tr>
<td>12.5</td>
<td>4</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>13.5</td>
<td>8</td>
<td>25</td>
<td>75</td>
<td>0</td>
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Table 2. Classification of homozygous mutant embryos at 9.5 dpc

<table>
<thead>
<tr>
<th>Class</th>
<th>Frequency (n)</th>
<th>Anterior truncations (%)</th>
<th>Trunk/posterior reduction (%)</th>
<th>Neural tube closure defect (%)</th>
<th>Ventral closure defect (%)</th>
<th>Axial rotation defect (%)</th>
<th>Reversal of heart looping (%)</th>
<th>Ballooning of pericardial sac; oedema (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>62% (24)</td>
<td>100</td>
<td>100</td>
<td>88</td>
<td>100</td>
<td>100</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>21% (8)</td>
<td>Variable</td>
<td>75</td>
<td>88</td>
<td>63</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>18% (7)</td>
<td>Variable</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>28</td>
<td>39</td>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 4. Scanning electron microscopy reveals defects in monocilium formation in mD2LIC–/– mouse embryos. (A) The distal tip of a wild-type embryo at 7.5-8.0 dpc (1-2 somite stage) viewed at low power. (C,E) The gross morphology of the node of homozygous mutant embryos is normal. Conditions and magnification are identical in A and C, and E is at twice the magnification. (B,G) Higher-power view of a wild-type embryo reveals rounded cells bearing monocilia (arrowheads). (D,H,F,I) Ventral node cells in mD2LIC–/– embryos are flatter than their wild-type counterparts and they lack normal monocilia. In some cases stunted structures are formed in the place of monocilia (arrows). G-I are at the same magnification. White boxes in B,D,F indicate regions of the node shown at higher magnification in G-I respectively. (F) Cells in the anterior region of the node and notochordal plate have the most extreme phenotype.

Fig. 5. Analysis of the mD2LIC phenotype at gastrula stages. (A,B) Expression of Fgf8 is unaffected by loss of mD2LIC function. (A) Lateral view of wild-type embryo; (B) Lateral view of mD2LIC–/– embryo. (C-E) Shh expression is reduced in the node and axial mesoderm of mD2LIC-null mutants. (C) Wild-type embryo showing expression of Shh in the node and in axial structures. (D,E) Reduction of Shh expression (arrowheads) in mD2LIC–/– embryos. (F-J) Reduction in Foxa2 expression in mD2LIC null mutant embryos. (F) Wild-type embryo showing expression of Foxa2 in the node and axial mesendoderm. (G,H) Expression of Foxa2 is severely reduced (G) or absent (H) in mD2LIC-null mutant embryos. (I,J) Foxa2 expression is later reduced in anterior definitive endoderm in mD2LIC–/– embryos. (I) Wild-type embryo. (J) mD2LIC–/– embryo. Arrowheads mark anterior definitive endoderm. (K-P) TUNEL analysis of mD2LIC–/– embryos reveals no elevation of cell death in the node or its derivatives but apoptosis does occur in anterior definitive endoderm. (K-M) Little apoptosis is observed in the embryonic region of wild-type embryos at 7.5 dpc (K, lateral view; L, anterior view; M, distal view). In mD2LIC–/– embryos, no apoptosis is observed in the node or its derivatives (P) but substantial cell death occurs in the anterior definitive endoderm (arrowheads, N, lateral; O, anterior).
left-right asymmetry is not generated correctly in mD2LIC−/− embryos, perhaps owing to the impairment of ciliogenesis and subsequent disruption of Nodal signalling, together with impaired development of the midline (see Discussion).

Anterior truncations and neural tube closure defects in mD2LIC−/− mutants

The later phenotypes observed in mD2LIC null embryos, such as anterior truncations and defects in neural tube closure, are likely to be consequences of the earlier defects. For example, expression of Foxa2 in the anterior definitive endoderm of mD2LIC−/− mutants is greatly reduced (Fig. 5I,J), and this is accompanied by extensive cell death in this tissue (Fig. 5K-P). mD2LIC is not normally expressed in the anterior definitive endoderm, suggesting that these cells are deprived of a survival signal that is normally produced by the node or its derivatives. During normal development, the anterior definitive endoderm maintains an Fgf8 signalling centre in the anterior neural ridge, which in turn is required for correct anterior development (Martinez Barbera et al., 2000; Shimamura and Rubenstein, 1997). Fgf8 expression in the anterior neural ridge is significantly reduced in mD2LIC−/− mutants (Fig. 6R,S), presumably because of defects in the anterior definitive endoderm, and the decrease in Fgf8 will lead to defects in anterior development (Fig. 7).

Defects in neural tube closure are also likely to be an indirect
Table 3. Analysis of notochord development in mD2LIC<sup>−/−</sup> mutant embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Wild type</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(notochord not always distinguishable)</td>
<td>(notochord not always distinguishable)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.8%</td>
<td>30.8%</td>
<td>19.3%</td>
<td>10.2%</td>
</tr>
<tr>
<td>2</td>
<td>35.3%</td>
<td>51.4%</td>
<td>43.5%</td>
<td>42.4%</td>
</tr>
<tr>
<td>3</td>
<td>59.9%</td>
<td>17.8%</td>
<td>41.2%</td>
<td>47.4%</td>
</tr>
</tbody>
</table>

Early notochord development can be subdivided into three stages: (1) formation of the notochord plate; (2) condensation of the notochord plate to form the notochord; and (3) separation of the notochord basement membrane from the endoderm. To characterise the notochord defect in detail, sections from between the level of the heart to the tail bud of 9.5 dpc mD2LIC<sup>−/−</sup> embryos were examined. These sections were obtained from representative embryos of all three classes, in order to determine how notochord development differs between the different classes of null mutants. Sections were stained by in situ hybridisation for expression of Nodal, Fgf8, and T. These structures and other aspects of the mD2LIC<sup>−/−</sup> phenotype are summarised in Fig. 7 and discussed below.

Discussion

The first detectable defect in mouse embryos lacking mD2LIC is a failure of ciliation formation in the node (Fig. 4). The normal assembly of cilia involves two types of intraflagellar transport. During anterograde transport, protein particles are translocated to the tip of the growing cilium; this process requires the kinesins Kif3a and Kif3b in a heterotrimeric complex with kinesin-associated protein 3 (Cole et al., 1998; Morris and Scholey, 1997). The particles are conveyed back to the base of the cilium by retrograde transport, and as we discuss below, it is this process that requires cytoplasmic dyneins such as mD2LIC.

Evidence implicating cytoplasmic dyneins in retrograde transport comes from work in Chlamydomonas and Caenorhabditis elegans. Chlamydomonas lacking either dynein 2 heavy chain (DHC2) or dynein light chain (LC8) fail most significantly, reduced expression of Shh will prevent the notochord from acting as a normal signalling centre, thereby interfering with formation of the floorplate and with expression of Foa2, Shh and Ebaf. In addition, however, TUNEL analysis at 9.0-9.5 dpc reveals that mD2LIC<sup>−/−</sup> embryos exhibit reduced levels of cell death in the hindbrain and also that there is ectopic cell death in the cephalic mesenchyme (Fig. 6T-Y). The elevated levels of apoptosis in the latter tissue may deprive the closing neural tube of its required mechanical support.

These and other aspects of the mD2LIC<sup>−/−</sup> phenotype are summarised in Fig. 7 and discussed below.
to undergo normal ciliogenesis, and any cilium-like structures that do form are reabsorbed (Pazour et al., 1999; Pazour et al., 1998). mD2LIC can associate with DHC2 (Grisson et al., 2002; Perrone et al., 2003), suggesting that this cytoplasmic dynein is also involved in retrograde transport. Consistent with this idea, ciliogenesis is impaired in C. elegans lacking XBX-1, a homologue of D2LIC, and as in the Chlamydomonas mutants, those cilia that do form subsequently disappear, perhaps through reabsorption (Schafer et al., 2003). Together, these results suggest that loss of mD2LIC in the mouse node is directly responsible for the observed defects in cilium formation.

**How does defective ciliogenesis lead to the mD2LIC<sup>−/−</sup> phenotype?**

As discussed below, several aspects of the mD2LIC mutant phenotype may be due directly to the loss of cilia and a disruption of nodal flow. Other defects, however, such as the early downregulation of genes such as Foxa2 and Shh, may have a more indirect aetiology. There may, for example, be a general impairment of cellular function: D2LIC is present in the Golgi apparatus and centrosomes (Grisson et al., 2002), and its loss may cause defects in protein maturation, in the cytoskeleton or in cell polarity.

Other elements of the mD2LIC<sup>−/−</sup> phenotype may result from a reduction in Hedgehog activity. The more severely affected mD2LIC<sup>−/−</sup> embryos resemble those deficient in Hedgehog signalling, such as the Smo<sup>−/−</sup> single mutant and Shh<sup>−/−</sup>/Ihh<sup>−/−</sup> double mutants (Zhang et al., 2001). mD2LIC<sup>−/−</sup> individuals also resemble embryos lacking nt, rotatin or St, as well as chimeric and conditional Foxa2<sup>−/−</sup> embryos (Dufort et al., 1998; Faisst et al., 2002; Hallonet et al., 2002; Izraeli et al., 1999; Melloy et al., 1998). Like mD2LIC<sup>−/−</sup> embryos, many of these mutants lack midline expression of Foxa2, one consequence of which would be a downregulation of Shh (Filosa et al., 1997).

It is possible that some of the later elements of the mD2LIC<sup>−/−</sup> phenotype are also consequences of defects in the Hedgehog signal transduction pathway. The phenotypes of our least severe ‘Class III’ embryos resemble those of embryos lacking the intraflagellar transport proteins Kif3a, Wim, Polaris and Fxo, in all of which the neural tube fails to close in the region of the head (Huangfu et al., 2003; Takeda et al., 1999). This is a characteristic of embryos that lack Shh (Huangfu et al., 2003) and it is possible that mD2LIC<sup>−/−</sup> individuals, such as Wim<sup>−/−</sup>, Polaris<sup>−/−</sup> and Fxo<sup>−/−</sup> embryos, are defective in Shh signal transduction as well as in Shh expression.

Finally, and as mentioned above, the lack of nodal cilia in mD2LIC<sup>−/−</sup> mice would be expected to interfere with nodal flow, and therefore with specification of the left-right axis. In the nodal flow hypothesis (Nonaka et al., 1998), cilia have been suggested to cause the unidirectional flow of a morphogen that thereby accumulates on just one side of the node and activates gene expression in an asymmetric fashion. More complicated models involve two populations of node monocilia, in which one population generates a fluid flow and the other senses and transduces it, thereby leading to an asymmetric calcium signal at the left-hand-side of the node (McGrath et al., 2003; Tabin and Vogan, 2003).

The absence of nodal cilia would disrupt asymmetric gene expression and thereby set in train the series of events that cause the defects we observe in mD2LIC<sup>−/−</sup> embryos. These events are described in Fig. 7, and include the decreased or symmetrical expression of genes such as Nodal, as well as the downregulation of T, Foxa2 and Shh. They culminate in the requirement for mD2LIC in the survival of the anterior definitive endoderm and thereby for maintenance of the anterior neural ridge and for normal anterior development. Models involving maintenance of the anterior neural ridge by anterior definitive endoderm have been proposed previously (Camus et al., 2000; Hallonet et al., 2002), although this is the first time that defects in axial mesendoderm have been shown to lead to cell death in the anterior definitive endoderm.

**Regulation of mD2LIC**

Recent work has demonstrated that expression of mD2LIC is downregulated in Rfx3-deficient mouse embryos, and that these embryos too show defects in cilium development and left-right axis specification (Bonnafe et al., 2004). Expression of mD2LIC<sup>−/−</sup> is not completely absent in Rfx3<sup>−/−</sup> individuals, however, and the phenotype of such mice is not as severe as that of our mD2LIC mutants, suggesting that other proteins also regulate mD2LIC expression.

This work was supported by the Medical Research Council and is dedicated to Rosa Beddington – much loved and missed. The authors thank members of the Divisions of Mammalian Development, Developmental Biology and Developmental Neurobiology, especially Simon Bullock, Jonathan Cooke, Sally Dunwoodie, Alex Gould, Shankar Srinivas and Derek Stemple, for helpful discussions. We are also grateful to Melanie Clements for technical assistance and to the staff of the Dunkin Green building for looking after the mice.

**References**


