Zebrafish curly up encodes a Pkd2 ortholog that restricts left-side-specific expression of southpaw

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The zebrafish mutation curly up (cup) affects the zebrafish ortholog of polycystic kidney disease 2, a gene that encodes the Ca\(^{2+}\)-activated non-specific cation channel, Polycystin 2. We have characterized two alleles of cup, both of which display defects in organ positioning that resemble human heterotaxia, as well as abnormalities in asymmetric gene expression in the lateral plate mesoderm (LPM) and dorsal diencephalon of the brain. Interestingly, mouse and zebrafish pkd2\(^{-/-}\) mutants have disparate effects on nodal expression. In the majority of cup embryos, the zebrafish nodal gene southpaw (spaw) is activated bilaterally in LPM, as opposed to the complete absence of Nodal reported in the LPM of the Pkd2-null mouse. The mouse data indicate that Pkd2 is responsible for an asymmetric calcium transient that is upstream of Nodal activation. In zebrafish, it appears that pkd2 is not responsible for the activation of spaw transcription, but is required for a mechanism to restrict spaw expression to the left half of the embryo. pkd2 also appears to play a role in the propagation of Nodal signals in the LPM. Based on morpholino studies, we propose an additional role for maternal pkd2 in general mesendoderm patterning.

KEY WORDS: Zebrafish, pkd2, Asymmetry, Left-right axis, Kidney, Pronephros, Laterality, Cilia

INTRODUCTION

The vertebrate body plan is organized into three primary body axes: anterior-posterior (AP), dorsal-ventral (DV), and left-right (LR). Although patterning along the AP and DV axes is readily observable, the internal asymmetries of the left-right axis are masked by an external bilateral symmetry. Instead, it is the asymmetric placement of internal organs that distinguishes the left and right sides. Human laterality disorders arise from defects in left-right patterning and exist in two primary forms: situs inversus and heterotaxia. Situs inversus, characterized by a complete reversal of the viscera, occurs at a rate of about 1 in 20,000 people and is associated with an increased incidence of congenital heart disease (CHD) (Mercola and Levin, 2001; Ramsdell, 2005). Heterotaxia, where one or more organs are positioned incorrectly, can have catastrophic effects on the development of the organism. Approximately 1 in every 8,000 births will result in heterotaxia, which also often manifests as CHD (Mercola and Levin, 2001).

The information necessary for the proper positioning of the viscera is provided by earlier asymmetric events including the left-side-specific expression of Nodal-related signaling factors. In the mouse embryo, asymmetric activation of Nodal transcription occurs at the ventral node during gastrulation. The node is lined by an epithelium that has one primary cilium protruding from the surface of each cell. Although cilia located at the node have 9+0 axonemes and are generally thought to be immotile, the presence of Left-right dynein (Ird; Dnahc11 – Mouse Genome Informatics) is thought to enable the central population of cilia to rotate in a clockwise direction. This ciliary rotation drives a leftward fluid flow over the node that is necessary for the proper establishment of sitedness, as mutations that compromise ciliogenesis or inhibit the mobility of these cilia result in randomization of the viscera (McGrath and Brueckner, 2003). In teleosts, Kupffer’s vesicle is thought to be analogous to the mouse node in the establishment of left-right patterning (Amack and Yost, 2004). A conserved role for ciliary flow in the establishment of left-right asymmetry is supported by motile cilia and flow found at Kupffer’s vesicle in zebrafish and Medaka embryos (Essner et al., 2005; Kramer-Zucker et al., 2005; Okada et al., 2005). Ciliary flow in the mouse has also been shown to be upstream of an asymmetric calcium flux in the epithelial cells adjacent to the left side of the node (McGrath and Brueckner, 2003), an event that appears to be conserved in chick and zebrafish (Raya et al., 2004; Sarmah et al., 2005). This left-specific intracellular calcium release is thought to be elicited by the flow-induced stretch activation of the calcium channel, polycystic kidney disease 2 (Pkd2), which localizes to the cilia of the mouse node (McGrath et al., 2003).

Human PKD2 encodes a six-pass transmembrane, Ca\(^{2+}\)-activated, non-specific cation channel, termed polycystin 2 (PC2) (Gonzalez-Perrat et al., 2001; Hanaoka et al., 2000; Vassilev et al., 2001). Heterozygous PKD2 mutations are found in approximately 15% of human autosomal dominant polycystic kidney disease patients (Wu et al., 1997). Mouse embryos homozygous for an inactivated allele of Pkd2 exhibit severe cardiac and kidney malformations and are embryonic lethal between E13.5 and parturition (Wu et al., 2000). Pkd2\(^{-/-}\) mice also display laterality disturbances in Nodal-related signaling genes, embryonic turning, visceral organs and heart morphogenesis. Most critically, without PC2 activity, no left-specific calcium expression is detected at the node and asymmetric activation of Nodal transcription does not occur, resulting in right isomerism (McGrath et al., 2003; Pennekamp et al., 2002). Therefore, current models for the initiation of a left-right symmetry breaking event include a role for PC2 upstream of asymmetric Nodal gene transcription. The link connecting this calcium transient with Nodal activation in the mouse is still unclear, although evidence exists for the involvement of the Notch pathway (Raya et al., 2004). In this paper, we present evidence for an alternative role of pkd2 in the development of the left-right axis.

Here we describe our analysis of curly up (cup), which encodes the zebrafish ortholog of polycystic kidney disease 2. We characterized two alleles of cup, and even though one allele of cup appears to be a null, cup mutants do not display defects in kidney
patterning nor do they develop kidney cysts. cup alleles display left-right defects in organ positioning that resemble human heterotaxia, as well as abnormalities in asymmetric gene expression in the lateral plate mesoderm (LPM) and dorsal diencephalon of the brain. Surprisingly, nodal expression patterns in the LPM are different in the mouse and zebrafish pkd2 mutants. In the majority of cup embryos, spaw is activated bilaterally in LPM. Although defects in the development of the notochord can result in bilateral transcription of nodal, we find that the midline structures in cup embryos are not compromised. Thus, in zebrafish, PC2 is not responsible for the activation of spaw transcription but is required for a mechanism to bias spaw expression towards the left half of the embryo.

MATERIALS AND METHODS

Zebrafish strains
cup9035b and cup5506b were obtained from a large-scale ENU mutagenesis screen (Brand et al., 1996; Haffter et al., 1996). Both cup mutations were generated in the TU strain and maintained by outcrossing to AB, WIK, and PWT. PWT is a wild-type strain generated in our laboratory that has a very low background of left-right defects. The cup phenotypes and differences in severity between cup9035b and cup5506b have been observed in multiple generations.

Positional cloning of curly up

Mapping of the cup locus was performed with SSLP markers as described (Liao and Zon, 1999). The cup genomic interval was narrowed between markers z10888 and z25625 on chromosome 1. Sequence surrounding these SSLP markers was identified through the Ensembl Sanger Sequence Database. Additional genomic sequence from the region was identified using BAC ends and BAC mate pair analysis to identify overlapping clones and assemble a genetic contig surrounding the cup locus. New SSLP markers used to narrow the cup genomic interval were created based on the identified genomic sequence: z9697.9 and z11984.6 (z9697.9F, 5′-GTCCTCGCT- TTGGTGTCAGAC-3′; z9697.9R, 5′-CTTCTCGATGCACAAGTCCG-3′; z11984.6F, 5′-GCGATCTCCAGAGAAACCAC-3′; z11984.6R, 5′-GTCCTGATCAGCAGCGG-3′). The following SNP primers were used to confirm linkage with pkd2: pkd2LSNPf, 5′-GATGTCATTGGCGGATCT- CGT-3′; pkd2LSNPr, 5′-CACACCTAAAGACGATCCGT-3′.

Sequencing of cup9035b and cup5506b and cloning and RT-PCR of pkd2

The zebrafish pkd2 gene was amplified from a 24-hour cDNA library created with the Marathon cDNA Amplification Kit (BD Biosciences) using cusp5B2f1 (5′-CAGATGCATGTATGAGGGCTTCG-3′) and cusp3pmel (5′-TCTTACGGTTTTAACTCACAAGTGGGCGGGGC-3′). The PCR product was cut with BglII and PmeI, and inserted into the BglII and EcoRV sites of vector T7TS (Cleaver et al., 1996). From production of an in situ hybridization probe, a BglII-Apal fragment from the T7TS-pkd2 plasmid was cloned into pBluescript II SK (+) using the BanHI and Apal sites. Full-length sequence was obtained by 5′ and 3′ RACE. Mutations in cup9035b and cup5506b were identified by sequencing genomic DNA.

RT-PCR of the pkd2 transcript was performed with total RNA isolated from 1-cell, 256-cell, 1-cell, sphere, and 18-somite stage embryos. cDNA libraries were made with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and primers located in exon 3 and exon 7 (pkd2 1F, 5′-GTGGGAGGCCAACAACTCTC-3′; pkd2 2R, 5′-CTTGAAGCGCCA-GCCACGCGCT-3′) were used to amplify a 700 bp fragment of pkd2.

RNA probes and whole-mount in situ hybridization

DIG-labelled RNA probes were transcribed from linearized DNA templates and used in RNA in situ hybridization by standard methods. Antisense probes included cardiac myosin light chain (cmcl2; myl7—ZFIN) (Yelon et al., 1999), forkhead 2 (fkh2; foxa3—ZFIN) (Odenthal and Nusslein-Volhard, 1998), nepreprosasin (nprp) (Milewski et al., 1998), southpaw (spaw) (Ahmad et al., 2004), paired-like homeodomain transcription factor (pita2) (Yan et al., 1999), polycystic kidney disease 2 (pkd2), sonic hedgehog (shh) (Krauss et al., 1993; Thieue and Thieue, 1999), lefty1 (Bisgrove et al., 1999), lefty2 (Bisgrove et al., 1999), SRY-box containing gene 17 (sox17) (Alexander and Stainier, 1999), alpha-tropomyosin (Ohara et al., 1989), orthodenticle homolog 5 (otx5) (Gamse et al., 2002), leftover (lov; kctd12.1—ZFIN) (Gamse et al., 2003), and charon (char) (Hashimoto et al., 2004).

Immunofluorescence

Embryos were fixed in Dent’s fixative at 4°C overnight, gradually rehydrated into PBBD (1×PBS, 0.1% Tween 20, 1% DMSO), and blocked for 2 hours in PBS containing 10% normal goat serum (NGS). To visualize cilia, embryos were incubated overnight at 4°C in a 1:40 dilution of monoclonal acetylated tubulin primary antibody (Sigma #T6793) in PBBD. Six 30-minute washes in PBBD containing 1% NGS and 0.1 M NaCl were administered before the last 30-minute wash in PBBD containing 1% NGS. The embryos were incubated overnight at 4°C with FITC-lgG2b at 1:500 (Southern Biotech #1090-02). The following day, the embryos were soaked in Hoechst (Molecular Probes #H3570) for 15 minutes at 1:1250 in PBBD before six additional 30-minute washes with PBBD containing 1% NGS and 0.1 M NaCl. After washing, the embryos were rinsed and stored in Slow Fade buffers (Molecular Probes #S-7461).

Microscopy

Images of five embryos were taken using the ProgressC14 digital camera (Jenoptik) mounted on a Leica MZFL III microscope. Embryos processed for immunofluorescence were mounted in Aqua-PolyMount (Polysciences #18606) and visualized on a Zeiss LSM 510. Embryos processed for in situ hybridization analysis were mounted in modified GMM (Struhl, 1981) [100 ml Canada Balsam (Sigma #C-1795), 10 ml methylsalicylate (Sigma #M0387-100G)], visualized using a Leica DMRA microscope at 10× magnification, and photographed with the ProgressC14 digital camera.

Morpholino injection

Morpholino antisense oligonucleotides (MO) (Gene Tools, LLC) were maintained in 50 µg/µl stock solutions in water at –80°C. The cup augMO, 5′-AGCTCATGTATTTCACAGTAA-3′, spans a portion of the 5′ UTR directly upstream of the AUG start site and the start site itself. The pkd2 augMO [previously reported as b1466 MO by Sun et al. (Sun et al., 2004), 5′-AGGACGAACGCGCATGGACCTCATC-3′, begins at the start AUG and extends into the first exon. Although these two MOs overlap by 8 nucleotides, only the pkd2 augMO effectively phenocopied cup (1-4 ng). The 9697-S1 splice-site MO, 5′-GAACCGGCTTCTGTGAACT-3′, is complimentary to the intron 3-exon 4 splice junction. The 9697-S2 splice-site MO, 5′-TAAACATACGACGTGTCATTG-3′, overlaps the exon 4-intron 4 splice junction of the pkd2 transcript. Only 9697-S1, used at 9 ng per embryo, was successful in phenocopying cup. Although this MO is predicted to block splicing, no alterations in the pkd2 message were observed by RT-PCR. Thus, we believe the 9697-S1 MO is more likely to be blocking translation. In support of this, other splice-site MOs have been shown to block translation of the message (M. Mullins, personal communication), but formal proof will require the generation of antibodies that recognize the Cup protein.

MOs were mixed with 5 mg/ml Phenol Red and injected into 1- to 4-cell stage embryos as described (Gritsman et al., 1999).

Histological analyses

Embryos were fixed in 4% paraformaldehyde (Sigma P6148) in PBS overnight at 4°C, and stored in 4% sucrose in PBS. After a gradual dehydration into ethanol, embedding was performed according to the Electron Microscopy Sciences protocol for JB-4 histology (EMS #14270-00). The embryos were sectioned on a Leica RM2255 Rotary Microtome at 0.1 mm Canada Balsam (Sigma #C-1795), 10 ml methylsalicylate (Sigma #M0387-100G), embedded according to the Leica MZFL III microscope. Embryos processed for in situ hybridization were mounted in modified GMM (Struhl, 1981) [100 ml Canada Balsam (Sigma #C-1795), 10 ml methylsalicylate (Sigma #M0387-100G), visualized using a Leica DMRA microscope at 10× magnification, and photographed with the ProgressC14 digital camera.

RESULTS

Mutations in the gene curly up affect pkd2

To gain insight into the genetic events involved in establishing the left-right axis, we have analyzed curly up, a mutation with left-right patterning defects that was originally isolated from a large-scale zebrafish mutagenesis screen as having a tail curl phenotype (Haffter et al., 1996; Development 134 (8))
The zebrafish *pcd2* transcript is 3.5 kb in length and encodes a 904 amino acid protein that is the zebrafish homolog of polycystin 2 (PC2) (Fig. 1B). PC2 is a Ca²⁺-sensitive cation channel with six hydrophobic stretches and intracellular N- and C-termini (Gonzalez-Perrett et al., 2001; Hanaoka et al., 2000; Vassilev et al., 2001). Zebrafish *pcd2* shares 67% and 65% identity, and 78% and 79% similarity, with the human and mouse orthologs, respectively (over 900 and 899 amino acid alignments; data not shown). Similarities include signaling elements such as an EF-Hand and a coiled-coil domain that are located in the C-terminus of the protein (Delmas et al., 2004).

To verify that *pcd2* is mutated in *cup*, each Ensembl-predicted exon was sequenced from *cup* genomic DNA of two different alleles, *cup*<sup>tc321</sup> and *cup*<sup>ty30b</sup>. Comparisons between the sequence of wild-type sibling embryos and *cup*<sup>tc321</sup> embryos identified a nonsense mutation in exon 2 that is predicted to generate a truncated protein consisting of only the N-terminal 135 residues (Fig. 1B). *ty30b* embryos have a missense mutation in exon 5 that changes a T to a C at nucleotide 1052, substituting a proline for a leucine in the first extracellular loop of the protein (Fig. 1B). We originally defined *cup*<sup>tc321</sup> as a stronger allele than *cup*<sup>ty30b</sup> based on the severity of the tail curl phenotype (Fig. 1C). Since *tc321* is likely to be a molecular null based on its predicted protein product, the mutations for each allele are consistent with *tc321* being a stronger loss-of-function allele than *ty30b*. The mouse anti-PC2 antibodies p57 and 58 recognize an antigenic region that includes the conserved leucine residue mutated in *ty30b* embryos. These antibodies can block PC2-dependent intracellular calcium release from ER stores (Nauli et al., 2003), and we therefore predict that a mutation in this region of the protein would interfere with proper channel activity.

et al., 1996). We utilized positional cloning techniques to identify the gene affected in the *cup* mutant. The *cup* mutation was placed on chromosome 1 using bulk segregant analysis (Talbot and Schier, 1999). Subsequent analysis was performed on 213 meiotic events to narrow the chromosomal location of *cup* to a 6.6 cM interval between simple sequence-length polymorphism (SSLP) markers z1088 and z25625 (Liao and Zon, 1999). We assembled a set of genomic clones that span the *cup* locus using available genomic sequence from the Sanger sequencing project. New SSLP markers were then created from these contig sequences (z9697.9 and z11984.6) and used to further refine the location of the *cup* mutation to a 2.6 cM region. One likely candidate for *cup* in this region was the gene affected in the *cup* mutant based on its predicted protein product, the mutations for each allele are consistent with *tc321* being a stronger loss-of-function allele than *ty30b*. The mouse anti-PC2 antibodies p57 and 58 recognize an antigenic region that includes the conserved leucine residue mutated in *ty30b* embryos. These antibodies can block PC2-dependent intracellular calcium release from ER stores (Nauli et al., 2003), and we therefore predict that a mutation in this region of the protein would interfere with proper channel activity.
Table 1. Defects in visceral and brain asymmetries in cup mutant embryos

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Situs solitus (%)</th>
<th>Situs inversus (%)</th>
<th>Heterotaxia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>97.0</td>
<td>0.0</td>
<td>3.0</td>
</tr>
<tr>
<td>cup&lt;sup&gt;tc321&lt;/sup&gt;</td>
<td>97</td>
<td>35.0</td>
<td>33.0</td>
<td>32.0</td>
</tr>
<tr>
<td>cup&lt;sup&gt;y30b&lt;/sup&gt;</td>
<td>98</td>
<td>37.8</td>
<td>45.9</td>
<td>16.3</td>
</tr>
<tr>
<td>augMO (2ng)</td>
<td>54</td>
<td>53.7</td>
<td>35.2</td>
<td>11.1</td>
</tr>
<tr>
<td>SpMO (9ng)</td>
<td>23</td>
<td>39.1</td>
<td>26.1</td>
<td>34.8</td>
</tr>
</tbody>
</table>

Viscera and brain data were collected at 48 hpf and 72 hpf, respectively. MOs were injected between the one- and four-cell stage. The laterality defects associated with 2 ng of pkd2 augMO are less severe than those in embryos injected with a higher dosage, but more general patterning defects can be seen at higher MO concentrations.

Table 2. Alterations in asymmetric gene expression in cup mutant embryos

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>n</th>
<th>spaw L (%)</th>
<th>spaw B (%)</th>
<th>spaw R (%)</th>
<th>spaw A (%)</th>
<th>ptx2 L (%)</th>
<th>ptx2 B (%)</th>
<th>ptx2 R (%)</th>
<th>ptx2 A (%)</th>
<th>lefty1/2 L (%)</th>
<th>lefty1/2 B (%)</th>
<th>lefty1/2 R (%)</th>
<th>lefty1/2 A (%)</th>
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<tbody>
<tr>
<td>LPM</td>
<td>Wild type</td>
<td>90</td>
<td>93.3</td>
<td>5.6</td>
<td>0.0</td>
<td>1.1</td>
<td>116</td>
<td>95.7</td>
<td>3.4</td>
<td>0.9</td>
<td>0.0</td>
<td>27</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>cup&lt;sup&gt;tc321&lt;/sup&gt;</td>
<td>114</td>
<td>73.7</td>
<td>20.2</td>
<td>6.1</td>
<td>0.0</td>
<td>192</td>
<td>63.6</td>
<td>16.2</td>
<td>3.6</td>
<td>114</td>
<td>69.3</td>
<td>1.8</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>cup&lt;sup&gt;y30b&lt;/sup&gt;</td>
<td>102</td>
<td>57.8</td>
<td>29.5</td>
<td>2.9</td>
<td>9.8</td>
<td>168</td>
<td>64.9</td>
<td>15.5</td>
<td>19.6</td>
<td>95</td>
<td>57.9</td>
<td>3.1</td>
<td>7.4</td>
</tr>
<tr>
<td>Brain</td>
<td>Wild type</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>116</td>
<td>94.8</td>
<td>1.7</td>
<td>0.9</td>
<td>2.6</td>
<td>27</td>
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<td>0.0</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>169</td>
<td>58.0</td>
<td>5.3</td>
<td>9.5</td>
<td>27.2</td>
<td>95</td>
<td>50.5</td>
<td>2.1</td>
</tr>
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</table>

L, left; B, bilateral; R, right; A, absent.

The mutant data above reflect the defects seen in clutches of embryos from a cross of tc321 and ty30b heterozygote parents. Therefore, the large percentage of left-sided expression of spaw and ptx2 is due to the approximate 75% of the population of embryos that are wild type or heterozygotes. Percentages of left-sided expression that are less than 75% may be attributed to errors in staging.

**Developmental results**

The expression of cup was found to be asymmetrically distributed, with a clear bias towards the left side. This bias was observed in the development of various organs, including the heart, liver, and pancreas. The expression pattern of cup was determined using RNA in situ hybridization with markers such as cmlc2, fkd2, and cmlc2. The results showed that cup expression was predominant on the left side, indicating a left-right asymmetry in the development of these organs.

**Discussion**

The asymmetric expression of cup suggests an early role in the establishment of left-right asymmetry during development. The data indicate that left-right asymmetry in organ development is present early in development, as evidenced by the expression of cup. This suggests that cup may play a role in the establishment of left-right asymmetry in the zebrafish embryo.

**Conclusion**

The study provides evidence for the involvement of cup in the establishment of left-right asymmetry in the zebrafish embryo. Further studies are needed to understand the molecular mechanisms underlying this process and to elucidate the role of cup in the development of asymmetry in other organisms.
Thus, in the zebrafish embryo, \textit{pkd2} spaw cup somitogenesis was proceeding normally (data not shown). Since mutant embryos at 8 somites and 24 hpf, indicating that \textit{pkd2}-deficient embryos (data not shown) \textit{is} expressed in the notochord and floorplate of the midline and \textit{cup} expression is present only in the notochord. Both gene products showed restricting because we observe a high percentage of additional role in the propagation of \textit{nodal} signals in this tissue.

\textit{pkd2} spaw lefty1 are also transcribed asymmetrically at 24 somites in the zebrafish brain (Essner et al., 2005). \textit{cup} embryos exhibited abnormal expression in the brain with the majority of embryos showing a loss of expression of these \textit{nodal} target genes (Fig. 3E-H, Table 2). Thus, \textit{pkd2} may play an additional role in the propagation of \textit{nodal} signals in this tissue.

\textit{pits2} and \textit{lefty1} are also transcribed asymmetrically at 24 somites in the dorsal diencephalon of the zebrafish brain (Essner et al., 2005). \textit{cup} embryos exhibited abnormal expression in the brain with the majority of embryos showing a loss of expression of these \textit{nodal} target genes (Fig. 3E-H, Table 2). Thus, \textit{pkd2} may play a role in the zebrafish diencephalon in sending or receiving an asymmetric signal from the LPM.

**Expression analysis of \textit{pkd2}**

To determine where \textit{pc2} activity is required for proper left-right patterning, expression patterns of \textit{pkd2} were analyzed by RNA in situ hybridization. Whereas mouse \textit{pkd2} is expressed ubiquitously (Pennekamp et al., 2002), we found distinct expression domains for \textit{pkd2} in zebrafish. Transcription of \textit{pkd2} was first detected at the onset of epiboly in cells marking dorsal fates at dome stage (Fig. 4B). Transcript levels increased as gastrulation continued, with expression expanding into the dorsal marginal cells (Fig. 4D,F).

Since transcripts cannot be detected at dome stage and at 40% epiboly in \textit{MZoep} embryos, which lack most of the mesendoderm, \textit{pkd2} is expressed in mesodermal and endodermal precursor cells during gastrulation (Fig. 4C,E). \textit{pkd2} also localized to the shield and dorsal forerunner cells (DFCs) (Fig. 4F,J). The DFCs maintained expression of \textit{pkd2} as they migrated towards the posterior, leading to a concentrated area of expression at the tailbud (Fig. 4G,K). These transcripts began to become more diffuse as the DFCs formed Kupffer’s vesicle. \textit{pkd2} mRNA was present in a ring-like pattern outlining the vesicle during early somite formation, but became less visible as somitogenesis proceeded. A low level of expression could be seen in the neural floorplate and pronephric duct primordia during these later stages (Fig. 4H,L).

Although not visible by RNA in situ hybridization, a maternal contribution of \textit{pkd2} mRNA was detected by RT-PCR at 1-4 cells and 256 cells, both of which are stages prior to the mid-blastula transition (1000 cells) (Fig. 4A). Since Kupffer’s vesicle has been implicated in left-right patterning in zebrafish, we predict that \textit{pc2} might be acting in this structure to affect left-right patterning (Amack and Yost, 2004; Essner et al., 2005). While this manuscript was in preparation, Bisgrove et al. found that injection of morpholinos against \textit{pkd2} to specifically knockdown translation of \textit{PC2} in Kupffer’s vesicle resulted in left-right patterning defects, supporting a role for \textit{pc2} in this location (Bisgrove et al., 2005). \textit{cup} embryos do have a structurally intact Kupffer’s vesicle as seen by light microscopy during mid-somitogenesis, and \textit{soxl7} expression highlights the proper migration of DFCs towards the tailbud region in mutants during late epiboly stages (data not shown). RNA in situ hybridization for \textit{charon}, a known Nodal inhibitor expressed at the start site (augMO), and one directed against an intron-exon junction (MO 9697-S1). Alterations in organ positioning and asymmetric \textit{spaw} expression were examined in order to assess the left-right patterning abnormalities in \textit{pkd2} morphants. Although the morphants displayed slightly milder tail curling (Fig. 5A-D), the laterality defects associated with the MO knockdowns were comparable to those of \textit{cup} mutants (Table 4). Interestingly, \textit{pkd2} morphants also exhibited hydrocephalus and dilations in the pronephric region, neither of which was displayed in

**Fig. 3. curly up affects expression of left-specific genes in the lateral plate mesoderm (LPM) and dorsal diencephalon.** Dorsal views of \textit{spaw} (A-D) and \textit{lefty1/2} (E-H) expression in 20- to 22-somite stage embryos (A-H) and ventral views of \textit{pits2} expression in the dorsal diencephalon of 24-somite stage embryos (I-L) from \textit{cup} heterozygote crosses. Alterations from normal left asymmetric Nodal signaling in the LPM (A) in \textit{cup} mutants include bilateral (B), right (C), and absent (D) expression of \textit{spaw} in this tissue. Over half of the bilaterally expressing embryos show only posterior propagation of \textit{spaw}. Similarly, asymmetrically expressed downstream targets of Nodal signaling in the LPM and brain are also disrupted. \textit{lefty1/2}, which are normally expressed in the left cardiac LPM (E), have bilateral (F), right (G), and absent (H) expression patterns in \textit{cup} mutants. In H, midline expression of \textit{lefty1} can also be seen, \textit{pits2}, which is normally expressed on the left side of the diencephalon (I), can be expressed bilaterally (J), on the right (K), or not at all (L) in \textit{cup} embryos. The majority of embryos show no expression of \textit{lefty1/2} in the LPM and diencephalon and no expression of \textit{pits2} in the diencephalon.
cup mutants (Fig. 5E,L). A number of genes known to cause cystic kidney phenotypes in other model organisms when mutated have been shown to cause pronephric cysts in zebrafish mutants and morphants (Kramer-Zucker et al., 2005; Liu et al., 2002; Otto et al., 2003; Sun and Hopkins, 2001), and MOs to \( pkd2 \) have been reported to cause kidney cysts (Sun et al., 2004). Thus, the finding that the pronephric region is dilated in our \( pkd2 \) morphants is not unexpected. However, we note that the dilations in our morphants are more elongated and are more restricted to the glomerulus than those seen in other pronephric mutants and morphants in the laboratory, and may not be representative of true pronephric cysts (our unpublished data). In fact, in \( cup \) mutants, severe edema is observed by day 6 in all tissues, suggestive of a general loss in fluid homeostasis not restricted to the pronephros or neural tube.

Table 3. Expression of \( lefty \) in cardiac LPM correlates with expression of \( spaw \) in anterior LPM

<table>
<thead>
<tr>
<th>( spaw ) expression</th>
<th>( n )</th>
<th>Left</th>
<th>Right</th>
<th>Bilateral</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior and posterior expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>83</td>
<td>79</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Right</td>
<td>4</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Bilateral (equal)</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Bilateral (left anterior; right posterior)</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Bilateral (right anterior; left posterior)</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Posterior expression only</td>
<td></td>
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<td></td>
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<tr>
<td>Left</td>
<td>0</td>
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<td>Right</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Bilateral (equal)</td>
<td>18</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>18</td>
</tr>
</tbody>
</table>

In situ hybridization for \( lefty1/2 \) and \( spaw \) were performed simultaneously in a clutch of \( cup^{+1 \beta} \) embryos. Embryos with expression of \( spaw \) in the area of the heartfield are scored as having anterior expression.

Fig. 4. Expression analysis of \( pkd2 \). (A) RT-PCR of \( pkd2 \) at early developmental time-points shows presence of maternal \( pkd2 \) prior to mid-blastula transition at 1K stage. \( gapdh \) was used to control for variations in total RNA for each cDNA library. Maternal contribution of \( pkd2 \) RNA cannot be detected by in situ hybridization. (B-E) Lateral views of \( pkd2 \) expression patterns in wild-type (B,D) and MZoep (C,E) embryos; anterior is up. Expression of \( pkd2 \) is first noticeable at dome stage, where \( pkd2 \) expression marks the dorsal side of the embryo (arrowhead in B) and continues to be expressed in all dorsal marginal cells by 40% epiboly (D). \( pkd2 \) expression is in mesendodermal tissue as expression is lost in MZoep embryos (C,E). (F,J) \( pkd2 \) RNA is detected at the shield in gastrulating embryos, as well as in the dorsal forerunner cells (DFCs, arrowhead in F and J; F anterior view, dorsal to the right; J is a higher magnification dorsal view focusing on the DFCs). (G,K) At tailbud stage, \( pkd2 \) is detected in a circular patch of cells that will soon form Kupffer’s vesicle (arrowhead in G and K; G lateral view, anterior is up, dorsal to the left; K is a higher magnification posterior view of region in G denoted by the arrowhead). (H,I,L) Dorsal view of 8-somite (H) and 36-hpf (I) embryos and cross-section view of an 8-somite embryo (L). Faint \( pkd2 \) expression highlighting the pronephric collecting ducts is marked with arrows and the more intensely stained floorplate in the midline is marked with arrowheads.
expression were evident at the lowest concentration tested (1 ng was analyzed. Left-right defects in organ placement and the dependence of these phenotypes on...

Additionally, curly up encodes zebrafish *pkd2*

Additionally, cup morphants have defects in body size and organ morphology, suggesting that the MOs cause more global organ patterning defects that could be the cause of the pronephric dilations we observe (see below).

Since cup<sup>tc321</sup> is likely to be a molecular null, the additional phenotypes seen in the *pkd2* morphants were somewhat unexpected.

To ensure that the N-terminus of PC2 that might be present in *cup*<sup>tc321</sup> embryos would be unable to account for these phenotypic differences, we supplied 9697-S1 splice morphants with the N-terminal fragment of *pkd2* upon injection at the 1-4 cell stage. The N-terminus of PC2 was unable to rescue any of the additional phenotypes in these morphants (data not shown). Another possibility is that the MOs are removing the maternal contribution of *pkd2*, leading to a more severe phenotype. To determine if the additional phenotypes we observe are indeed due to a more severe loss of *pkd2*, the dependence of these phenotypes on *pkd2* augMO concentration was analyzed. Left-right defects in organ placement and *spaw* expression were evident at the lowest concentration tested (1 ng augMO per embryo), but the curly tail phenotype did not appear until 2 ng of augMO per embryo (Table 4 and data not shown). Dilations in the pronephric area occurred in a low percentage of embryos at this concentration, but increased in frequency with increasing MO concentration (Table 4). Hydrocephalus was only present in a small population of morphants that had been injected with 4 ng of *pkd2* augMO (Table 4). However, at this concentration general body defects were observed, including narrowed axes, smaller heads and overall loss of proper organ development. The pancreas and liver were often positioned on the midline and much smaller than in wild-type embryos (Fig. 5I). The glomerulus of the kidney was also often indiscernible in sectioned embryos (Fig. 5I). Thus, left-right patterning is most sensitive to decreases in *pkd2* levels, followed by the tail curl and dilation phenotypes, respectively. Overall, these results suggest that zygotic *pkd2* is required for left-right patterning and proper tail elongation, whereas loss of maternal *pkd2* results in more severe body defects, perhaps owing to mispatterning in the mesendoderm where *pkd2* is expressed.

cup mutants display no obvious structural ciliary defects

Primary cilia exist on the cells that line the ventral node of the mouse embryo. PC2 localizes to these cilia and has been shown to be responsible for eliciting an asymmetric calcium surge in the epithelial cells adjacent to the left side of the node (McGrath et al., 2003). Cilia also reside on the cells of Kupffer’s vesicle in the zebrafish embryo (Essner et al., 2002). To determine if cilia are affected in *cup* morphant and mutant embryos, an antibody against acetylated tubulin was used to visualize cilia by immunofluorescence. As the curly tail phenotype cannot be seen in *cup* mutants until 33 hpf, *pkd2* augMO morphants were analyzed at 12 somites for the presence of cilia on Kupffer’s vesicle. The cilia at Kupffer’s vesicle in *pkd2* morphants appeared structurally intact (Fig. 6A,B), as did the cilia lining the collecting ducts of the kidney in *cup* mutants at 33 hpf (Fig. 6C,D). Because the formation of Kupffer’s vesicle is unaffected in *pkd2* mutants and morphants, it was not surprising to see the same circular distribution of cilia in the morphant embryos. Although no obvious structural abnormalities are detected in the cilia of *pkd2* mutant zebrafish, we cannot rule out the possibility that the cilia might be functionally compromised.

DISCUSSION

The requirement for *pkd2* in left-right patterning is conserved, but the role of *pkd2* might not be

In this study, we identify *pkd2* as the gene affected in the zebrafish *cup* mutant. *pkd2* encodes the cation channel PC2, one of two proteins that are mutated in human polycystic kidney disease (Wu et al., 1997). Similar to the *Pkd2* knockout mouse, *cup* mutants exhibit a variety of...

| Table 4. Morpholino knockdown of PC2 results in laterality, kidney and mesendoderm defects in a concentration-dependent manner |
|---|---|---|---|---|---|---|
| *pkd2* augMO | n | Situs solitus (%) | Situs inversus (%) | Heterotaxia (%) | Abnormal (%) | n | Dilation (%) |
| Control | 45 | 100.0 | 0.0 | 0.0 | 0.0 | 29 | 0.0 |
| 1 ng | 66 | 63.6 | 27.3 | 9.1 | 0.0 | 38 | 5.3 |
| 2 ng | 54 | 53.7 | 35.2 | 11.1 | 0.0 | 44 | 65.9 |
| 3 ng | 84 | 25.0 | 42.9 | 6.0 | 26.1 | 53 | 60.4 |
| 4 ng | 56 | 0.0 | 0.0 | 0.0 | 100.0 | 46 | 97.8 |

Injections were performed between the one- and four-cell stage in a 5 mg/ml Phenol Red solution. Control injections were with Phenol Red solution alone. The abnormal visceras category includes endoderm defects ranging from multiple liver and pancreata, to thinner body axes that only allowed for midline and improperly shaped abdominal organs. At 4 days, the injected embryos had what appeared to be cystic pronephric regions that differed in size and shape as compared with our normal definition of a kidney cyst. Therefore, we refer to this phenotype as a kidney dilation. Embryos injected with 2 ng or more of the augMO had upward curvatures in their body axes.
organ laterality defects, supporting a conserved requirement for \( \text{pkd2} \) function in left-right patterning. In \( \text{pkd2} \) mutant zebrafish, \( \text{spaw} \) and \( \text{pitx2} \) are both expressed bilaterally in the LPM, and the majority of \( \text{spaw} \) expression is restricted to the posterior. \( \text{lefty} \) expression is absent in the cardiac LPM in the majority of \( \text{cup} \) embryos, although we believe the absence of asymmetric \( \text{lefty} \) is due to the inability of \( \text{spaw} \) to propagate in the LPM in embryos lacking \( \text{PC2} \) (Fig. 7). We also find discordance in the percentage of embryos expressing bilateral \( \text{spaw} \) and right-sided \( \text{pitx2} \) in \( \text{cup} \) mutants. We attribute these differences to the following possibilities. Since \( \text{spaw} \) expression is often uneven in its anterior propagation in \( \text{cup} \) mutants, the side that receives the \( \text{spaw} \) signal more prevalently will be the side to express \( \text{pitx2} \). Alternatively, although \( \text{spaw} \) is thought to act directly upstream of \( \text{pitx2} \), it is possible that \( \text{spaw} \) is not the sole regulator of \( \text{pitx2} \) in the LPM during these stages.

In mouse, the proposed role for \( \text{PC2} \) in activation of Nodal signaling is a prominent feature of the ‘two-cilia’ model. In this model (reviewed by Tabin and Vogan, 2003), cilia-generated ‘nodal-flow’ is thought to bend non-motile cilia at the left edge of the node and activate \( \text{PC2} \). Flow activation of \( \text{PC2} \) leads to an increase in calcium at the left edge of the node, which results in activation of the Nodal signaling pathway on the left by an unknown mechanism. Aspects of this model are based in large part on the \( \text{Pkd2} \) knockout mouse, as calcium is not upregulated on the left side of the node and \( \text{Nodal} \) is not activated in the LPM (McGrath et al., 2003; Pennekamp et al., 2002). However, our work convincingly demonstrates that \( \text{spaw} \) is expressed in embryos lacking the \( \text{PC2} \) channel and suggests that the role of \( \text{PC2} \) is to restrict \( \text{spaw} \) to the left LPM.

The differences between \( \text{cup} \) and the \( \text{Pkd2}^{\text{null}} \) mouse with regards to \( \text{nodal} \) expression are significant and call into question the validity of strictly applying current models for \( \text{PC2} \) function in left-right patterning to zebrafish. It is clear that some aspects of the two-cilia model are valid in zebrafish, but others are not. For example, Kupffer’s vesicle is ciliated and there is a fluid flow in this vesicle, but the flow is reported to be circular, and does not have a left bias (Essner et al., 2005; Kramer-Zucker et al., 2005). A number of calcium asymmetries surrounding Kupffer’s vesicle have been reported (Sarmah et al., 2005; Webb and Miller, 2000). However, which calcium asymmetries may be relevant for the establishment of the left-right axis and how calcium asymmetries may function in this process are not clear.

One possible explanation for the differences between mouse and zebrafish is that the role for \( \text{pkd2} \) in left-right patterning is conserved, but the actual function the channel plays in each organism is not. This would be similar to the situation for \( \text{Fgf8} \), which has a conserved role in left-right patterning, but is reported to act as a left-determining factor in mouse and a right-determining factor in chick and rabbit (Boettger et al., 1999; Fischer et al., 2002; Meyers and Martin, 1999). It should be noted, however, that two out of 12 \( \text{Pkd2} \) mutant mice described by Pennekamp et al. exhibited bilateral expression of \( \text{Nodal} \).
in the LPM (Pennekamp et al., 2002). Intriguingly, a large percentage of the mouse mutants do exhibit bilateral Pitx2 expression, a downstream target of Nodal signaling, and the domain of expression is restricted to the posterior portion of the embryo (Pennekamp et al., 2002), similar to the posterior expression of spaw in cup mutants. Thus, it is fair to conclude that Nodal signaling can be initiated in the mouse in the absence of PC2, and that the bias of left-sided expression is now lost. This is similar to our findings in fish and suggests that the role of PC2 in left-right patterning in all organisms may need to be reconsidered.

In addition, the mechanism by which PC2 acts in any organism to affect asymmetric events is still unclear. In mouse, PC2 is thought to act at the node to affect left-right patterning. Zebrafish pkd2 is expressed in Kupffer’s vesicle, the proposed node-equivalent in teleosts, and Bisgrove et al. report a role for pkd2 in this structure (Bisgrove et al., 2005). A recent model proposes that activity in the mouse node provides a subtle asymmetry on the left side that manifests into robust expression of Nodal in the left LPM through a combination of activating signals on the left and repressive signals on the right (Nakamura et al., 2006). PC2 may be acting from within Kupffer’s vesicle to initially bias the activation of spaw gene expression to the left side of the organism. Alternatively, PC2 might be responsible for repressing Nodal signaling on the right half of the embryo (Fig. 7). Based on pkd2 expression data, this repression could take place at multiple sites in the embryo, including Kupffer’s vesicle and the floorplate. As no midline defect is apparent in cup mutants, we believe that it is unlikely that PC2 in the floorplate is responsible for restricting spaw to the left side, but this has not been formally proven. If pkd2 is acting at multiple levels to affect the direction of spaw activation and spaw propagation, Nakamura’s model (Nakamura et al., 2006) predicts that the majority of embryos would display bilateral expression of spaw, which is what we observe. This model also explains how alterations in Nodal initiation and propagation can result in right-sided or no expression of Nodal, which we observe in a low percentage of cup mutants.

Our results also suggest that maternal pkd2 might play a role in proper mesendoderm patterning of the visceral organs. Thus, it is possible that the effects of pkd2 on left-right patterning could occur prior to the formation of Kupffer’s vesicle. Interestingly, in Xenopus, there is evidence that indicates an earlier non-ciliary role for PC2 in left-right patterning prior to gastrulation (Qiu et al., 2005).

A possible role for Cup in mesendoderm patterning

We find that pkd2 is expressed maternally in zebrafish, and has restricted expression to mesendoderm during early epiboly and gastrulation. Our work with MOs suggests that pkd2 may play a role in the patterning of the mesendoderm. For example, at high doses of MO against pkd2, various mesendoderm defects are apparent, including loss of glomerular tissue in the kidney, smaller organs including the liver and gut, and thinner body axes. These defects are variable, but were seen with two different MOs against pkd2 and are thus likely to be specific defects. The Pkd2 knockout mouse also displays pancreatic, liver, kidney and cardiac complications at early developmental stages, further supporting a function for PC2 in mesoderm and endoderm (Wu et al., 2000).

Although low levels of MO against pkd2 phenocopied the left-right defects seen in zygotic cup mutants, we believe cup mutants are a better system for studying the role of pkd2 in left-right patterning. In fact, while this manuscript was in preparation, another study was published on pkd2 using MOs in zebrafish that came to different conclusions on the role of pkd2 in left-right patterning (Bisgrove et al., 2005). We believe that the differences in our conclusions might be due in part to the additional effects of the pkd2 MO on the mesendoderm, which are not seen in zygotic cup mutants.

Similarly, zygotic cup embryos do not develop pronephric cysts during larval development as would be predicted for a mutation in a gene that causes polycystic kidney disease in other vertebrates. Although we could detect dilatations in the pronephric region of wild-type embryos injected with a high concentration of pkd2 augMO, histological sections revealed that the cystic tissue had an unconventional morphology as compared with other cystic mutants in the laboratory (our unpublished data). We believe the MO-specific phenotype in the kidney is due to the knockdown of maternal pkd2 mRNA that is present in zygotic cup mutants, similar to what has been proposed previously (Sun et al., 2004), but because mesendoderm defects are apparent in pkd2 morphants, the cystic kidney phenotype is questionable in its origin. Although MO knockdown of pkd2 could be affecting redundancies in pkd2 or alternative splice forms, we have found no evidence for the existence of these to date. We attempted to analyze protein knockdown in mutants and morphants using several antibodies against human and mouse PC2, but none of these antibodies were able to cross-react with the zebrafish protein as assessed by whole-mount in situ hybridization or western blotting. Future studies on whether maternal pkd2 mRNA is affecting early mesendoderm patterning will be needed to fully understand the dilated kidney phenotype we observe in the morphants.

Organ laterality defects in cup mutants suggest multiple levels of regulation in left-right patterning

Visceral organ patterning is affected in cup mutants and can be categorized into three main sub-groups: normal organ positioning, situs inversus, and heterotaxia. It is intriguing that each phenotypic group constitutes about one-third of the total population of cup mutants, and we see similar segregation in other asymmetry mutants in our laboratory (our unpublished results). It has been proposed that in the absence of asymmetric signaling, each organ will randomly adopt a position on the left-right axis resulting in a high degree of heterotaxia (Burdine and Schier, 2000; Concha et al., 2000; Yan et al., 1999). The fact that such a large population of mutants with altered spaw signaling retains either wild-type or completely inverted patterning is unexpected and suggests some global left-right information is still retained when asymmetric Nodal signaling is disrupted. This idea is further supported by the finding that whereas over 30% of cup mutant embryos show complete situs inversus, only a small percentage of them show right-sided spaw expression. We have shown that the expression of downstream asymmetric genes depends on the magnitude of spaw expression on each side, and thus it is important to incorporate the expression patterns of multiple Nodal pathway signals in a model of how asymmetric nodal genes translate into the proper positioning of the organs.

cup mutants also show a loss of asymmetric gene expression in the dorsal diencephalon of the brain at 24 somites and randomized asymmetric placement of the parapineal and dorsal habenula. These data are consistent with the phenotypes of other mutants that lack Nodal signaling in the zebrafish brain (Concha et al., 2000). cup embryos are unique, though, in their ability to activate Nodal pathway gene expression in the LPM but not in the diencephalon of most mutants. It is intriguing to speculate that the activation of nodal
genes in the brain might also be dependent on the anterior propagation of spaw in the LPM, or upon pkd2 activity in the diencephalon.

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References
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