Differential regulation of node formation, nodal ciliogenesis and cilia positioning by Noto and Foxj1

Leonie Alten1, Karin Schuster-Gossler1, Anja Beckers1, Stephanie Groos2, Bärbel Ulmer3, Jan Hegermann4, Matthias Ochs4 and Achim Gossler1,*

SUMMARY

The mouse transcription factor Noto is expressed in the node and controls node morphogenesis, formation of nodal cilia and left-right asymmetry. Noto acts upstream of Foxj1, which regulates ciliogenesis in other mouse tissues. However, the significance of Foxj1 for the formation of cilia in the mouse node is unclear; in non-amniote species Foxj1 is required for ciliogenesis in the structures equivalent to the node. Here, we analyzed nodes, nodal cilia and nodal flow in mouse embryos in which we replaced the Noto-coding sequence with that of Foxj1, or in embryos that were deficient for Foxj1. We show that Foxj1 expressed from the Noto locus is functional and restores the formation of structurally normal motile cilia in the absence of Noto. However, Foxj1 is not sufficient for the correct positioning of cilia on the cell surface within the plane of the nodal epithelium, and cannot restore normal node morphology. We also show that Foxj1 is essential for ciliogenesis upstream of Rfx3 in the node. Thus, the function of Foxj1 in vertebrate organs of asymmetry is conserved, and Noto regulates node morphogenesis and the posterior localization of cilia on node cells independently of Foxj1.

KEY WORDS: Mouse node, Cilia, Left-right determination

INTRODUCTION

The node is a transient structure at the anterior end of the primitive streak of amniote vertebrate embryos that is essential for embryo patterning (reviewed by Camus and Tam, 1999; Viebahn, 2001). In mouse embryos, the node becomes morphologically visible around embryonic day (E) 7.5 at the distal tip of the embryo as a shallow, crescent-shaped depression on the endodermal side. At this stage, the node constitutes the posterior extreme of the forming notochord (PNC) (Blum et al., 2007), the ventral cell layer of which faces the outer curvature of the embryo with its apical side. Hereafter, we refer to the node/PNC as ‘node’ for simplicity. Each ventral node cell carries a monocilium that protrudes into the extracellular space on its apical surface (Sulik et al., 1994). The core of a cilium, the so-called axoneme, consists of a stereotypically arranged set of microtubules that originates at the so-called basal body, which is anchored in the cortical actin cytoskeleton. The basal body of a primary cilium is a centrosomal structure consisting of a mother centriole, which serves as the organizing center for the axonemal microtubules, and a closely attached perpendicular daughter centriole (reviewed by Nigg and Raff, 2009). Nodal cilia are motile, but in general their axonemes contain only nine peripheral doublet microtubules and lack the central microtubule pair that is characteristic for motile cilia (Feistel and Blum, 2006; Satir and Christensen, 2007). However, some nodal cilia with a central microtubule pair were also observed (Caspary et al., 2007). Monocilia in the central node rotate clockwise. This rotation, in combination with the posterior localization of the cilium on the cell surface and the posteriorly tilted rotational axis, leads to the generation of a leftward nodal flow of extra-embryonic fluid (Nonaka et al., 1998). This flow generates a symmetry-breaking signal by mechanically stimulating sensory monocilia on cells unilaterally in the periphery of the node (McGrath et al., 2003; Shiratori and Hamada, 2006) or by establishing a gradient of a secreted morphogen (Nonaka et al., 1998; Okada et al., 2005). Ultimately, this signal is translated into the asymmetry of visceral organs. Mutations that affect the formation and structure (Nonaka et al., 1998; Marszalek et al., 1999; Takeda et al., 1999; Murcia et al., 2000; Taulman et al., 2001; Huangfu et al., 2003; Huangfu and Anderson, 2005; Houde et al., 2006), sensory function (Pennekamp et al., 2002) or motility (Supp et al., 1997; Watanabe et al., 2003) of monocilia in the node disrupt the normal generation of left-right asymmetry, demonstrating their essential role in this process.

Noto is the mouse member of the Not family of homeobox genes. Not genes are expressed in the node of amniotes, or in Kupffer’s vesicle in bony fish and the gastrocoel roof plate in amphioxans, which represent the homologous structures to the murine node (von Dassow et al., 1993; Knezevic et al., 1995; Stein and Kessel, 1995; Talbot et al., 1995; Abdellahalek et al., 2004; Plouhinec et al., 2004). In mouse embryos, Noto is required in the node and for normal notochord formation in and posterior to the lower trunk region (Abdelkhalek et al., 2004). Left-right determination in the embryo depends on Noto in the node, where it regulates node morphogenesis and ciliogenesis. Embryos lacking Noto display nodes with highly variable and irregular shapes and sizes. Cilia in Noto mutant nodes are short, and basal bodies are often detached from the apical cell surface. In Noto mutants, several genes important for cilia function or assembly are downregulated, and cilia have disorganized or incomplete...
axonomal microtubules (Beckers et al., 2007). Among the genes directly or indirectly regulated by Noto are those encoding the transcription factors Foxj1 and Rfx3, which are expressed in the node and in many other tissues containing ciliated cells (Reith et al., 1994; Blatt et al., 1999; Brody et al., 2000). FOXJ1 and RFX3 regulate the expression of a number of genes important for cilia structure and function and directly activate an overlapping set of target genes (reviewed by Thomas et al., 2010). Loss of RFX3 leads to short but ultrastructurally normal nodal cilia, and causes left-right defects (Bonnafe et al., 2004). Likewise, loss of FOXJ1 causes left-right defects, although apparently normal cilia were observed in the node, whereas cilia are absent from multi-ciliated lung epithelial cells (Chen et al., 1998; Brody et al., 2000). In the absence of Noto, Foxj1 and Rfx3 are downregulated in the node. This raises several questions: which of the defects in Noto mutant nodes are caused by reduced Foxj1 and Rfx3 expression; which of these defects are direct consequences of loss of NOTO and which are mediated by an as yet unidentified factor; and what is the regulatory relationship between Foxj1 and Rfx3. To address these questions, we generated mice expressing Foxj1 instead of Noto and analyzed nodes and nodal cilia in these embryos and in embryos deficient for Foxj1. Our analyses indicate that Rfx3 acts downstream of Foxj1 in the node and that Foxj1 expression is necessary for the generation of motile nodal cilia. In the absence of functional Noto, Foxj1 is sufficient to restore motile cilia in the node, and Noto functions independently of Foxj1 in node morphogenesis and polarized localization of cilia in the node.

MATERIALS AND METHODS

Mouse lines and genotyping

To express Foxj1 instead of Noto we replaced GFP in the targeting vector used to generate the NotoGFP null allele (Abdelkhalek et al., 2004) by the Foxj1 coding sequence followed by IRES2-ttdomato and a neo cassette flanked by frt sites. Gene targeting, ES cell screening and chimera production was carried out as described for the NotoGFP allele. The neo cassette was excised by crossing germ line chimeras to FLPdeletor mice (Rodriguez et al., 2000). The Noto Foxj1 allele after neo excision was genotyped by PCR using the primers Foxj1Ki-Neo-F1 (AGACCGAGATAGGGTTGAGTGTTG) and Δ-neo-B (GCACAACACACATAAAAGAGG); the wt and NotoGFP alleles were genotyped as described (Abdelkhalek et al., 2004). Foxj1lacZ mice (Brody et al., 2000) were genotyped by allele-specific PCR using primers HGT1 (CTTAAAGGCCAGATGGGAGAGG) and HGT4 (AGCCGTTGGGGTCTGTGC) resulting in a 519 bp wild-type product, and HGT1 and HGT-lacZ (CTCTCGTATTACGCCAGCTGG) resulting in a 416 bp product indicative of the mutant allele. All mouse work performed conformed to the regulatory standards for experimental animal work as set out by the legal authorities.

In situ hybridization

Mutant and wild-type embryos were processed in parallel under identical conditions by standard procedures (Beckers et al., 2007). Pictures were obtained as described previously (Beckers et al., 2007).

Direct video microscopy of nodal cilia

Motility of nodal cilia was analyzed as described (Schweickert et al., 2007).

Video microscopy and image analysis for nodal flow analysis

The flow of fluorescent beads (125-fold dilution of FluoSpheres, 0.5 μm, Molecular Probes in F10 culture Media, Gibco) in the cavity of the PNC/ventral node was recorded for durations of at least 60 seconds as described (Schweickert et al., 2007) at 10 frames per second (fps) and at 20X magnification using a Zeiss AxiosMot2 equipped with an AxioCam HSM video camera. Trajectories were visualized using ImageJ software in combination with the MTrackJ plugin (http://www.imagescience.org/meijering/software/mtrackj/). Time-lapse movies were analyzed and transformed into trajectories by ParticleTracker (Balzarini and Koumoutsakos, 2005) (ImageJ; http://rsb.info.nih.gov/ij/). Trajectories were processed by a custom-made script for project-R (http://cran.r-project.org/) as described (Schweickert et al., 2007; Maisonneuve et al., 2009).

Scanning electron microscopy

Embryos were dissected in ice-cold phosphate-buffered saline (PBS) and immediately fixed in 0.1 M sodium cacodylate buffer, pH 7.4, with 3% glutaraldehyde overnight followed by washes in 0.1 M sodium cacodylate buffer, post-fixation in 2% OsO4 in 0.1 M sodium cacodylate buffer and dehydration in a graded acetone series. Embryos were critical-point dried, mounted and sputter coated with gold. Analysis was performed using an SEM 505 microscope (Philips, Eindhoven, The Netherlands).

Transmission electron microscopy

Embryos were dissected in ice-cold PBS, immediately fixed (2.5% glutaraldehyde, 2% formaldehyde, freshly prepared from paraformaldehyde, 1.7 mM CaCl2 in 0.1 M sodium cacodylate-HCl buffer, pH 7.3) overnight, washed in 0.1 M sodium cacodylate with 0.2 M sucrose, post-fixed in 1% OsO4 in 0.1 M sodium cacodylate followed by washes in sodium maleate buffer, pH 5.2, contrastin in 2% uranylacetate in sodium maleate buffer, washes in sodium maleate buffer, dehydration in a graded ethanol series, incubation in Toloul, Toloul/Epon 1:1 and Epon, and embedding in Epon. Specimens were oriented such that sections were perpendicular to the proximo-distal axis. Polymerization was performed for 20 hours at 40°C followed by 40 hours at 60°C. Thin sections (~65 mm thick) were collected on Formvar-coated copper slot grids and stained with uranylacetate and lead citrate. Sections were selected at lower magnifications and investigated in detail using a transmission electron microscope (Tecnai 20, FEI Company, Eindhoven, The Netherlands) at an acceleration voltage of 200 kV. To exclude influences from the arbitrary section planes, sectioned cilia were observed with electron tomography at tilt angles ranging from −70° to +70° with an increment of 1°.

Immunohistochemistry, image acquisition and processing

Embryos were dissected in ice-cold PBS and fixed for 1 hour in DMSO/MeOH/H2O2 (4:1:1) on ice, subjected to three 5 minute washes and a 5 hour incubation in 50 mM NH4Cl-PBS, an 8 hour incubation in 0.1% H2O2/TS-PBS (1% Triton X-100, 10% FCS in PBS) and three 1 hour incubations in TS-PBS. Primary antibodies (anti-ZO1: Zymoted, #226-0031; anti-γ-tubulin: Sigma, #T 5326; anti-Dishevelled 2: abcam, #ab22616) were diluted 1:500 in TS-PBS and embryos were incubated for 3-5 days then subjected to three 1 hour washes in TS-PBS. Embryos were incubated with secondary antibodies (anti-rabbit Alexa 350, #A11046; anti-mouse Alexa 488, #A21202; anti-rabbit Alexa 488, #A21206; all Invitrogen), diluted 1:100 in TS-PBS for 16-20 hours, then washed three times in TS-PBS for 1 hour per wash. All steps were carried out at 4°C. Pictures were obtained with a Leica DMI6000B microscope and HCX PL APO 63×/1.40-0.60 Oil objective. Pictures were collected as z-stacks with 0.1 μm (DVL2) or 0.2 μm (ZO1/γ-tubulin) spacing. Stacks of 25 pictures were processed using the Leica LAS AF 2.3 software. Stacks were analyzed using the 3D Deconvolution tool (Method: Blind; Refractive Index: 1.518; Auto generate PSF; ten iterations) and the projection was generated using the 3D Projection tool.

qRT-PCR

Embryos were dissected in PBS and transferred into RNAlater (Ambion, #AM7020). DNA and RNA were isolated from individual age-matched embryos using TriReagent (Sigma, #T9424) according to the manufacturer’s protocol. cDNAs were synthesized from RNA samples from individual genotyped embryos using the ThermoScript RT-PCR System (Invitrogen, #11146-016) according to the manufacturer’s protocol. PCRs were performed with Platinum TaqPCRx DNA Polymerase (Invitrogen, #11509-015) according to the basic protocol with SYBR Green and ROX (Invitrogen, #12223-012) in a 25 μl reaction volume in a 7500 Fast Real-Time PCR System (Applied Biosystems) in duplicate (at least). Gene-specific primers were checked for near 100% amplification efficiency and used to amplify short fragments of Dynlrb2 (qDynlrb2-F1, qDynlrb2-R1).
AGACCTGAGCCAGGGAGCTCGCCACC; qDynhbr2-B1, AGGTTTCTCTCCATTCTGCTCATCG; qRfx3-F1, GACACGATACAGT-GATGGGTGAG; qRfx3-B4, TCCAGATTTCCGGGAGATACAGCAT), Nphp3 (qRfx3-F1, ATGAGGAAAAGACAGTGCCTATGGAATGCTCG; qNphp3-B4, TGCTGCCATATGGAATGCTCG) and Shh (qShh-F1, ATGAGGAAAAGACAGTGCCTATGGAATGCTCG; qShh-B1, AGATGGCAGACAGCACAGCAC). Ct-values were translated into fold change of expression levels using the ΔΔCt-method (Livak and Schmittgen, 2001). Shh, which is expressed in the node and in cells along the anterior midline and expression levels of which appeared to be unaffected in Noto or Foxj1 mutants by whole-mount RNA in situ hybridization (WISH), served as reference gene. Mutant probes were compared with wild-type probes and the average relative wild-type expression was defined as 1. For Rfx3, Nphp3 and Shh, a second independent PCR was performed, confirming the obtained results (data not shown).

RESULTS

Generation and characterization of Noto<sup>Foxj1/Foxj1</sup> mice

We generated a mouse that expresses functional FOXJ1 protein in the node in the absence of NOTO protein by knocking in the Foxj1 open reading frame (followed by an internal ribosome entry site and the red fluorescent marker tdTomato) into the endogenous start codon of Noto. This deleted most of Noto exon 1, intron 1 and exon 2, and disrupted Noto in a similar manner to the Noto<sup>GFP</sup> null allele that we described previously (Fig. 1A) (Abdelkhalek et al., 2004). Heterozygous mice carrying this allele (Noto<sup>Foxj1/Foxj1</sup>) were phenotypically normal (data not shown). Heterozygous E7.5-8 embryos expressed Nodal and tktin 2 (Tkt2), markers for crown and pit cells, respectively, indistinguishably from wild-type embryos (data not shown), and expressed tdTomato specifically in the node, recapitulating endogenous Noto expression in wild-type embryos (Fig. 1Ba,b). As expected, homozygous Noto<sup>Foxj1</sup> embryos (n=4) did not express Noto (Fig. 1Ce). Homozygous knock-in mice displayed shortened and kinked tails (Fig. 1Cl) that resembled the previously described Noto-null phenotype (Abdelkhalek et al., 2004), probably as a result of the lack of Noto function in the nascent notochord at later stages of development, when Noto, but not Foxj1, is expressed in wild-type embryos. In homozygous Noto<sup>Foxj1</sup> E7.5-8 embryos (n=5), Foxj1 mRNA was readily detected, in contrast to Noto-null mutants (n=5; Fig. 1Be-e). The axonemal dynein Dnahc11 is severely downregulated in homozygous Noto<sup>GFP</sup> mutants (n=4; Fig. 1Db) (Beckers et al., 2007) and is a known FOXJ1 target in cells of the respiratory epithelium (Chen et al., 1998). Transgenic expression of Foxj1 restored expression of Dnahc11 in homozygous Noto<sup>Foxj1</sup> embryos (n=4) at E7.5-8 (Fig. 1Dc). Foxj1 mutant embryos (n=5) display abnormal expression of laterality genes (for example, see Fig. 1Dd). One copy of transgenic Foxj1 expressed from the Noto<sup>Foxj1</sup> allele restored the normal left-sided expression of Nodal and Lefty1 in 4-6 somite stage embryos (n=6) lacking endogenous FOXJ1 (Fig. 1De). Collectively, these data indicate that Foxj1 expressed from the Noto locus is functional and sufficient to drive expression of FOXJ1 targets in the node.
Noto regulates expression of cilia-associated
genases through Foxj1

We continued to analyze the consequences of Foxj1 expression on ciliogenesis in the node, node morphology and gene expression in the absence of NOTO. Because node morphology and cilia length in the node are stage dependent (Sulik et al., 1994; Bonnafe et al., 2004), embryos were staged according to Downs and Davies (Downs and Davies, 1993) and, if not indicated otherwise, all comparative analyses were done using age-matched embryos from different litters at the late head fold stage or at a slightly earlier stage we refer to as mid head fold stage (for examples, see supplementary material Fig. S1). The loss of Noto function led to reduced expression of several cilia-associated genes. For example, mRNA levels of Dynlrb2, which encodes a cytoplasmic dynein light chain; Dync2h1, which encodes the heavy chain of the cytoplasmic dynein-2 retrograde motor and is a component of retrograde intraflagellar transport (Huangfu and Anderson, 2005); Rfx3, a transcription factor required for normal cilia growth (Bonnafe et al., 2004); and Tekt2, a member of a family of microtubule-stabilizing proteins (Amos, 2008) were severely reduced or not detected in NotoGFP/GFP mutants (n≥4) by in situ hybridization (Beckers et al., 2007) (Fig. 2Ab,f,j,n). In homozygous NotoFoxj1 embryos, transcript levels of these genes were restored (n≥4), although it appeared that full wild-type levels might not be reached (Fig. 2Ac,g,k,o). mRNA quantification by qRT-PCR (exemplarily carried out for Dynlrb2 and Rfx3 on independent RNA prepared from at least five embryos per genotype; Fig. 3) confirmed the severe reduction in NotoGFP/GFP mutants and revealed an upregulation to almost wild-type levels in NotoFoxj1/Foxj1.

Foxj1 restores ciliogenesis and cilia motility in the absence of Noto

The restored expression of genes important for ciliogenesis in homozygous NotoFoxj1 nodes suggested that defective microtubule structure, shortening and immotility of cilia that were found in nodes lacking Noto (Beckers et al., 2007) might be rescued by the knock-in of Foxj1 into Noto. In sections from six NotoFoxj1/Foxj1 embryos, we observed 15 cilia, five of which could be evaluated unequivocally for the structure of axonemal microtubules by transmission electron microscopy and showed no microtubule defects (Fig. 2Bc), in contrast to the disrupted structure of axonemal microtubules in NotoGFP/GFP mutants that was described previously (Fig. 2Bb) (Beckers et al., 2007). Consistent with a previous report (Bonnafe et al., 2004), cilia length in wild-type nodes increased from the mid to late head fold stage (n=70 and 103 cilia, three and four embryos, respectively; Fig. 2Ca,D). At both stages, cilia length was significantly reduced in NotoGFP/GFP mutants (n=68 and 49, four and eight embryos, respectively; Fig. 2Cb,D), and partially restored in homozygous NotoFoxj1 embryos (n=58 and 99, three and five embryos, respectively; Fig. 2Cc,D).
Nodal cilia in wild-type embryos (n=7) rotate clockwise (supplementary material Movie 1), whereas in homozygous NotoGFP embryos (n=3) nodal cilia are essentially immotile (Beckers et al., 2007). Nodal cilia in homozygous NotoFoxj1 embryos (n=4) regained clockwise rotational motility (supplementary material Movie 2), indicating that Foxj1 expression in the node is sufficient to generate motile nodal cilia in the absence of NOTO.

**Foxj1** is an important regulator of ciliogenesis and cilia function in the murine node

Our finding that Foxj1 is sufficient to restore cilia structure and motility in the Noto-deficient node suggests that Foxj1 is also necessary for nodal ciliogenesis. This, however, is not consistent with previous scanning electron microscopic data that did not reveal obvious defects in nodal cilia in Foxj1-deficient embryos (Brody et al., 2000). To investigate this discrepancy, we analyzed expression of the above-mentioned cilia-associated genes as well as structure, length and motility of cilia in Foxj1−/− mutant embryos, carrying the previously analyzed Foxj1−/− allele (Brody et al., 2000). Transcripts of Dynlrb2, Dynch2h1, Rfx3 and Tek2 were not detected in Foxj1-null mutant nodes (n≥4) by in situ hybridization (Fig. 2Ad,h,l,p), and for Dynlrb2 and Rfx3 severe downregulation was confirmed by quantitative PCR of cDNAs from individual embryos (n=6; Fig. 3). This indicates that expression of genes important for ciliogenesis requires Foxj1 in the node and suggests that cilia formation and function might be abnormal. In sections from six Foxj1−/− embryos, we could unequivocally evaluate 14 transverse sections of cilia for the structure of axonemal microtubules by transmission electron microscopy. Four out of these showed obvious defects in the structure and arrangement of axonemal microtubules (Fig. 2Bd). The difficulty of obtaining more sections of cilia might, in part, be explained by the severe reduction in the length of Foxj1−/− mutant cilia that we observed in scanning electron microscopy. Cilia length was reduced to about one-third in both mid and late head fold stage embryos (n=72 and 100, four and five embryos, respectively; Fig. 2Cd,D), and was more pronounced than in NotoGFP/GFP mutants (Fig. 2Ch,D). In addition, cilia analyzed in Foxj1−/− mutants (n=4) by videomicroscopy were completely immotile (supplementary material Movie 3).

**Motile cilia in NotoFoxj1/Foxj1 mutant nodes are not sufficient to generate a leftward nodal flow and establish correct left-right asymmetry**

Despite the restored expression of ciliary components and wild-type-like rotational motility of cilia, homozygous NotoFoxj1 embryos displayed abnormal expression of laterality genes. In wild-type embryos, Nodal and Lefty1 are expressed in the left lateral plate mesoderm and along the midline at the 4-6 somite stage (Fig. 4Aa). In homozygous NotoFoxj1 embryos (n=7), these expression patterns were randomized. Nodal/Lefty1 expression was detected unilaterally (on either the left or the right), was symmetrically present or absent, or was expressed at different levels on both sides (for an example, see Fig. 4Ac), resembling the defective expression of laterality genes we previously described for NotoGFP/GFP mutants (Beckers et al., 2007) (Fig. 4Ab). One potential explanation could be that, despite rotational movement of cilia, no normal nodal flow is established in NotoFoxj1/Foxj1 embryos. To test this directly we observed the movement of fluorescent beads in the nodes of 2-4 somite-stage embryos by videomicroscopy. As a qualitative measure of flow, the mean resultant length of particle trails (Rayleigh’s test of uniformity) was calculated from each time-lapse movie and was expressed as the dimension-less number rho (ρ). A p-value of 1 corresponds to movement of all beads in the same direction and ρ=0 corresponds to random movement. In wild-type embryos (n=6), fluorescent beads moved to the left side of the node (Fig. 4Bae, supplementary material Movie 4) with a ρ of 0.62. Consistent with immotile cilia, there was essentially no directional movement of beads in NotoGFP/GFP (ρ=0.08; Fig. 4Bbf, supplementary material Movie 5) and Foxj1−/− (ρ=0.09; Fig. 4Bdh, supplementary material Movie 7) mutant embryos (n=8, respectively). Directional movement in NotoFoxj1/Foxj1 nodes (ρ=0.21; n=5; Fig. 4Bcg, supplementary material Movie 6) was slightly higher than in NotoGFP/GFP and Foxj1−/−, but clearly reduced compared with wild type. This supports the idea that abnormal nodal flow underlies the laterality defects in these embryos despite restored cilia length and motility.

**Noto is required for proper node morphogenesis**

Left-right defects can be caused by an irregular shape of the node (Lee et al., 2010). Because NotoGFP/GFP mutant embryos have irregular nodes of variable size and show abnormal expression patterns of genes delineating the node (e.g. Beckers et al., 2007) (Fig. 4Cf,n,Db,f,j), we analyzed node morphology in homozygous NotoFoxj1 embryos by scanning electron microscopy (n=8) and marker gene expression. These analyses showed that nodes in homozygous NotoFoxj1 embryos had variable shapes and sizes similar to nodes in NotoGFP/GFP embryos (compare Fig. 4Cf,n with 4Cg,o). This was also reflected by abnormal expression domains of marker genes for node cells. The area of Nphp3 (n=4) and Shh (n=6) expression was variable and compressed, and the width and shape of the crown cell region delineated by expression of Dand5 (n=3) was no longer horseshoe-shaped but was narrow and irregular (Fig. 4Dcg,k). By contrast, in Foxj1−/− mutant embryos (n=9), which retain Noto expression (supplementary material Fig. S2), the shape of the node (Fig. 4Ch,p), as well as the expression patterns of Nphp3 (n=5), Shh (n=4) and Dand5 (n=5) (Fig. 4Ddh,l) were essentially normal, although the nodes appeared somewhat smaller in some Foxj1−/− mutant embryos.
Defective polarized localization of nodal cilia in Noto\textsuperscript{GFP/GFP}, Noto\textsuperscript{Foxj1/Foxj1} and Foxj1\textsuperscript{–/–} mutant embryos

Effective nodal flow requires the polarized location of cilia in the plane of node cells towards the posterior end (Nonaka et al., 2005; Antic et al., 2010). To investigate whether mislocalization of cilia could also contribute to defective left-right patterning, we analyzed the position of cilia on node cells in scanning electron micrographs of embryos of the various genotypes. In wild-type nodes, in >75% of the cells (n=78/103 in four embryos) cilia were anchored at the posterior pole of the cell (Fig. 5Aa,Ba). By contrast, ~50% of the cilia in Noto\textsuperscript{GFP/GFP} (n=27/49 in eight embryos), Noto\textsuperscript{Foxj1/Foxj1} (n=49/99 in five embryos) and Foxj1\textsuperscript{–/–} mutants (n=50/100 in five embryos) emerged at a central position, the others at roughly equal rates anterior, posterior, left or right to the central position (Fig. 5Ab-d,Ba, supplementary material Table S1). To confirm independently that the position of cilia is randomized in mutant embryos, we stained embryos with antibodies against \(\gamma\)-tubulin, which marks centriolar structures including the basal bodies, and ZO1, which outlines the cell borders (Fig. 5 Ae-h). Images shown in j and n are reproduced with permission from Beckers et al. (Beckers et al., 2007). (D) Altered expression patterns of Nphp3, Shh and Dand5 in LHF stage embryos. Probes and genotypes are indicated to the left and at the top, respectively.

DISCUSSION

In Noto mutant embryos, node morphogenesis and nodal ciliogenesis are disrupted. Here, we have shown that expression of Foxj1 from the Noto locus is sufficient to restore the motility of...
cilia and the transcription of genes important for ciliogenesis in mutants lacking Foxj1 function. The rescue of gene expression and cilia structure in NotoFoxj/Foxj1 embryos is consistent with cilia defects in Foxj1–/– mutant embryos and indicates that in mouse embryos Noto regulates essential aspects of nodal ciliogenesis through Foxj1. In addition, we have provided evidence that Foxj1 is indispensable for the formation of functional motile cilia in the murine node. This finding is in line with Foxj1 function in Xenopus and zebrafish embryos. In these species, Foxj1 is required for the formation of cilia in the gastrocoel roof plate and Kupffer’s vesicle, respectively (Stubbs et al., 2008), which represent structures equivalent to the node.

We detected Rfx3 mRNA in the node only in the presence of Foxj1 expression (Fig. 2Ai-l). Thus, in node cells there appears to be a clear epistatic relationship between Foxj1 and Rfx3, with Foxj1 acting upstream of Rfx3. This differs from cultured multiciliated mouse ependymal cells, in which RFX3 regulated Foxj1 expression (El Zein et al., 2009), and from mouse floorplate cells, in which expression of Rfx3 was unaffected by the loss of Foxj1 (Cruz et al., 2010). Thus, the regulatory relationship between these two transcription factors that govern ciliogenesis appears to differ between the node and other tissues. It is noteworthy that ectopic expression of Foxj1 in neural progenitors converted short primary cilia into long cilia, but in Foxj1 mutant floorplate cells cilia length was unaffected, which was attributed to redundant functions of Rfx3 and Foxj1 (Cruz et al., 2010). In cultured mouse ependymal cells, loss of Rfx3 function led to reduced growth of motile cilia and affected expression of genes involved in cilia assembly and motility (El Zein et al., 2009). Similarly, in the mouse node, Rfx3 mutants have short but ultrastructurally normal cilia (Bonnafe et al., 2004) raising the possibility that reduced or absent Rfx3 expression in both Noto and Foxj1 mutants might underlie the ‘short cilia’ phenotype and defective motility in both mutants. NotoGFP/GFP mutants expressed Foxj1 at a low, but still detectable, level (Fig. 1Bd). Genes that were severely downregulated but still detected in NotoGFP/GFP mutants (Fig. 2Aj,n) were not detected in Foxj1–/– mutants (Fig. 2Al,p), and cilia length was less reduced in NotoGFP/GFP than in Foxj1–/– mutant nodes (Fig. 2D). Altogether, these defects in NotoGFP/GFP mutants appear to be slightly milder than those of Foxj1–/– mutants, which might be due to residual Foxj1 activity in NotoGFP/GFP mutants, and further supports the notion that Noto regulates crucial aspects of ciliogenesis through Foxj1 and Rfx3. By contrast, Foxj1 expression does not restore node morphogenesis in the absence of NOTO and Foxj1–/– embryos exhibit normal node morphology. These findings indicate that Noto regulates pivotal aspects of node morphogenesis independently of Foxj1, and suggest that the abnormal shape of nodes in NotoFoxj1/Foxj1 embryos, rather than undetected irregularities in the motion of NotoFoxj1/Foxj1 nodal cilia, contributes to the left-right defects. However, we cannot exclude the possibility that abnormal transcript levels of Foxj1 expressed from the Noto locus contribute to some of the remaining defects in NotoFoxj1/Foxj1 embryos.

Mutants lacking Noto or Foxj1 function showed randomized positions of cilia on the surface of node cells. This could indicate that both genes function together to localize cilia at the posterior cell pole. We cannot rule out this possibility, but the random positioning of cilia might also arise for different reasons in the different genotypes. In Foxj1 mutant airway epithelial cells, no cilia form, apical actin and ezrin localization is disrupted, and basal bodies are not anchored at the cell cortex (Huang et al., 2003; Gomperts et al., 2004; Pan et al., 2007). In the node of Foxj1–/– mutants, two out of seven detected centriolar structures (daughter centrioles or basal bodies without an emerging cilium in the plane of section), were located abnormally deep in the cytoplasm, and we found sections of a cilium emerging from such deep basal bodies were also observed in NotoGFP/GFP mutant embryos (Fig. 2Ei-b) (Beckers et al., 2007) but not in sections from NotoFoxj1/Foxj1 mutant nodes (Fig. 2Eh), although we cannot rule out the possibility that such cilia or basal bodies exist but were not observed. These observations raise the possibility that basal bodies are not correctly localized at the cell
cortex in Foxj1−/− mutant node cells, similar to airway epithelial cells, which might contribute to the abnormal cilia positioning in nodes lacking Foxj1. In NotoFoxj1/Foxj1 nodes, cilia might not be correctly localized for a different reason. In these mutants, expression of known Foxj1 targets but not Nphp3 (nephrocystin 3; nephronphthisis 3 – Mouse Genome Informatics) was restored (Fig. 3). NPHP3 protein interacts with inversin, a component of the planar cell polarity (PCP) signaling pathway, and is required for convergent extension cell movements in Xenopus embryos, suggesting a role in establishing planar cell polarity (Bergmann et al., 2008). Loss of Nphp3 function in mice disrupts normal left-right asymmetry (Olbrich et al., 2003; Bergmann et al., 2008). Thus, the reduced expression of Nphp3 in NotoGFP/GFP mutants raises the possibility that defective PCP signaling contributes to left-right defects by abnormal cilia positioning in the nodes of these embryos. However, it is unclear whether the observed reduction of Nphp3 levels in NotoGFP/GFP and NotoFoxj1/Foxj1 mutants is sufficient to cause PCP defects, because no such defects were described in heterozygous Nphp3 mutants (Bergmann et al., 2008). Although microarray analyses did not detect transcriptional downregulation of known PCP pathway components in NotoGFP/GFP mutants (L.A., A.B. and A.G., unpublished observation), we tried to detect potential alterations in the distribution of known PCP proteins in Noto mutant node cells. Although commercially available VANG1 and PRICKLE2 antibodies did not work reliably in our hands, DVL2 protein was consistently reduced at the apical membrane of node cells in NotoGFP/GFP and NotoFoxj1/Foxj1 mutants (n=5 each) compared with wild-type (n=6) and Foxj1−/− mutant (n=6) nodes (Fig. 5C). However, DVL genes function redundantly in the mouse node (Hashimoto et al., 2010), and it is unclear whether the localization and/or amount of other DVL proteins is also affected. Nonetheless, as basal bodies appear to be correctly attached at the apical membrane in NotoFoxj1/Foxj1 mutants, but fail to localize posteriorly, our data suggest that posterior localization of cilia is controlled by Noto in a Foxj1-independent manner.

In summary, our analysis has distinguished processes in the mouse node directly regulated by Noto from processes requiring Foxj1 and Rx3 function, and has established the regulatory relationship between Foxj1 and Rx3. Foxj1 is indispensable for functional nodal cilia, and acts upstream of Rx3. Noto regulates nodal ciliogenesis by means of Foxj1, but Noto is required for node morphogenesis independently of Foxj1, and establishes the posterior localization of cilia potentially by affecting planar cell polarity.

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