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

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**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-amilote 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 amnionic vertebrate embryos that is essential for embryonic 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 cilience, which serves as the organizing center for the axonemal microtubules, and a closely attached perpendicular daughter cilience (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 (Caspar 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 amphibians, 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; Murcia et al., 1999; Abdelkhalek et al., 2004; Plouiniec 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

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axonemal 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 (Abdelkhaled et al., 2004) by the *Foxj1* coding sequence followed by IRES2-tdTomato 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 FLPe deleter mice (Rodriguez et al., 2000). The *NotoFoxj1* allele after neo excision was genotyped by PCR using the primers Foxj1-Ki-Neo-F1 (AGACGAGATAGGGTGAAGTTGG) and Δ-neo-B (GGCAACCCACACTAATAAAGAGG); the wt and *NotoGFP* alleles were genotyped as described (Abdelkhaled et al., 2004). *Foxj1lacZ* mice (Brody et al., 2000) were genotyped by allele-specific PCR using primers HGT1 (TTCAGGCCAGATGAGGAGAGG) and HGT4 (AGCCGGTTGGGTCTCTTGTGC) resulting in a 519 bp wild-type product, and HGT1 and HGT-lacZ (CTCCTCGCTATATTGGGCGAGCTGTG) 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 (Schweikert 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 (Schweikert et al., 2007) at 10 frames per second (fps) and at 20× magnification using a Zeiss AxioMot2 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 (Balzarin and Kroumoutsakos, 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 (Schweikert et al., 2007; Mainsonneve 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 parafomaldehyde, 1.7 mM CaCl2 in 0.1 M sodium cacodylate-HCl buffer, pH 7.3) overnight, washed in 0.1 M sodium cacodylate with 0.22 M sucrose, post-fixed in 1% OsO4 in 0.1 M sodium cacodylate followed by washes in sodium maleate buffer, pH 5.2, contrasting in 2% uranylacetat in sodium maleate buffer, washed 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 nm 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 DML6000B 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,
AGACTTGAGCCAGGAGCCTGCCCAC; qDynlb2-B1, AGGGTTC-CTCAGAGCTCTCGTCATCQ; Rfx3 (qRFX3-3 F4, GACACGTAAGAGT-GAGGAGGAG; qRfx3-B4, TCCATTCGCGAGCATAAGCT), Nphp3 (qNph3-3 F4, GCGAGATGCAGCTGTCG; qNph3-B4, TGGCTGCTATGGAATGCTCG) and Shh (qShh-F1, AGATGGCC AGGGCA TTTA AC -TGCTGCCTATGGAATGCTCG) and Shh (qShh-F1, ATGAGGAAAA-)

RESULTS

Generation and characterization of Noto\textsuperscript{Foxj1/Foxj1} 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\textsuperscript{GFP} null allele that we described previously (Fig. 1A) (Abdelkhalak et al., 2004). Heterozygous mice carrying this allele (Noto\textsuperscript{Foxj1/+}) were phenotypically normal (data not shown). Heterozygous E7.5-8 embryos expressed Nodal and tektin 2 (Tekt2), markers for crown and pit cells, respectively, indistinguishably from wild-type embryos (data not shown), and expressed tdTomato specifically in the node, and pit cells, respectively, indistinguishably from wild-type embryos (Fig. 1Ba,b). As expected, homozygous Noto\textsuperscript{null} mice (n=5) failed to express Noto (Fig. 1Ce). Homozygous knock-in mice displayed shortened and kinky tails (Fig. 1Ci) that resembled the previously described Noto-null phenotype (Abdelkhalak 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\textsuperscript{null} E7.5-8 embryos (n=5), Foxj1 mRNA was readily detected, in contrast to Noto-null mutants (n=5; Fig. 1Bc-e). The axonemal dynein Dnahc11, a downstream effector of Noto and Foxj1, in LHF stage nodes (a-c) and representative examples of adult mice (b,d,f); tail length of homozygous Noto\textsuperscript{null} mice is highly variable, as reported for Noto\textsuperscript{GFP/GFP}. (D) Expression of Dnahc11, a downstream effector of Noto and Foxj1, in LHF stage nodes (a-c) and ventral views of Nodal/Lefty1 expression in 4-6 somite stage embryos (d,e). Genotypes are indicated at the top.

![Gene targeting strategy, functional validation of Foxj1 expressed from the Noto locus, and external defects of Noto\textsuperscript{Foxj1/Foxj1} mice.](image-url)
Noto regulates expression of cilia-associated genes 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 homozygous NotoFoxj1 embryos, transplant 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.

**Fig. 2. Restored expression of Noto and Foxj1 downstream effectors and partial rescue of cilia structure in NotoFoxj1/Foxj1 embryos.** (A) Ventral views of LHF stage embryos after WISH with probes as indicated to the left. At least four embryos per genotype and probe were used; representative examples are shown. (B) Transmission electron micrographs of nodal cilia of LHF stage embryos. Arrowheads in d point to disrupted or incomplete microtubules in Foxj1–/– mutants, which were also found in NotoGFP/GFP mutants. Scale bars: 100 nm. (C) Scanning electron micrographs of node cells of LHF stage embryos showing short cilia in Foxj1–/– and NotoGFP/GFP and restored cilia length in NotoFoxj1–/– mutants. Scale bars: 4 μm. (D) Length of nodal cilia in the indicated genotypes at the mid (a) and late (b) head fold stage. Error bars indicate s.d. P<0.0001. (E) Transmission electron micrographs of basal bodies in the node of LHF embryos. Scale bars: 200 nm. Genotypes are indicated at the top. Images (Bb and Eb) are reproduced with permission from Beckers et al. (Beckers et al., 2007).
Nodal cilia in wild-type embryos \((n=7)\) rotate clockwise (supplementary material Movie 1), whereas in homozygous \(\text{Noto}^{\text{GFP}}\) embryos \((n=3)\) nodal cilia are essentially immotile (Beckers et al., 2007). Nodal cilia in homozygous \(\text{Noto}^{\text{Foxj1}}\) embryos \((n=4)\) regained clockwise rotational motility (supplementary material Movie 2), indicating that \(\text{Foxj1}\) expression in the node is sufficient to generate motile nodal cilia in the absence of \(\text{NOTO}\).

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

Our finding that \(\text{Foxj1}\) is sufficient to restore cilia structure and motility in the \(\text{Noto}\)-deficient node suggests that \(\text{Foxj1}\) 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 \(\text{Foxj1}\)-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 \(\text{Foxj1}\)-null mutant embryos, carrying the previously analyzed \(\text{Foxj1}^{\text{null}}\) allele (Brody et al., 2000). Transcripts of \(\text{Dynlnb2}, \text{Dynlch2}, \text{Rfx3}\) and \(\text{Tekt2}\) were not detected in \(\text{Foxj1}\)-null mutant nodes \((n>4)\) by in situ hybridization (Fig. 2Ad,h,l,p), and for \(\text{Dynlnb2}\) and \(\text{Rfx3}\) 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 \(\text{Foxj1}\) in the node and suggests that cilia formation and function might be abnormal. In sections from six \(\text{Foxj1}^{\text{null}}\) 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 \(\text{Foxj1}^{\text{null}}\) 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 \(\text{Noto}^{\text{GFP/GFP}}\) mutants (Fig. 2Cb,D). In addition, cilia analyzed in \(\text{Foxj1}^{\text{null}}\) mutants \((n=4)\) by videomicroscopy were completely immotile (supplementary material Movie 3).

**Motile cilia in \(\text{Noto}^{\text{Foxj1/Foxj1}}\) 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 \(\text{Noto}^{\text{Foxj1}}\) embryos displayed abnormal expression of laterality genes. In wild-type embryos, \(\text{Nodal}\) and \(\text{Lefty1}\) are expressed in the left lateral plate mesoderm and along the midline at the 4-6 somite stage (Fig. 4Aa). In homozygous \(\text{Noto}^{\text{Foxj1}}\) embryos \((n=7)\), these expression patterns were randomized. \(\text{Nodal/Lefty1}\) 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 \(\text{Noto}^{\text{GFP/GFP}}\) 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 \(\text{Noto}^{\text{Foxj1/Foxj1}}\) 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 \((\rho)\). A \(p\)-value of 1 corresponds to movement of all beads in the same direction and \(p=0\) corresponds to random movement. In wild-type embryos \((n=6)\), fluorescent beads moved to the left side of the node (Fig. 4Ba,e, supplementary material Movie 4) with a \(p\) of 0.62. Consistent with immotile cilia, there was essentially no directional movement of beads in \(\text{Noto}^{\text{GFP/GFP}}\) \((p=0.08;\) Fig. 4Bb,f, supplementary material Movie 5) and \(\text{Foxj1}^{\text{null}}\) \((p=0.09;\) Fig. 4Bd,h, supplementary material Movie 7) mutant embryos \((n=8\), respectively). Directional movement in \(\text{Noto}^{\text{Foxj1/Foxj1}}\) nodes \((p=0.21; n=5)\;\text{and}\;\text{Foxj1}^{\text{null}}\) \((\rho<0.0001)\) was expressed as the dimension-less number \(\rho\) and \(\rho=1\) corresponds to movement of all beads in the same direction. A close scrutiny of these embryos revealed that nodal flow was not normal in \(\text{Noto}^{\text{GFP/GFP}}\) and \(\text{Foxj1}^{\text{null}}\) embryos, 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 \(\text{Noto}^{\text{GFP/GFP}}\) 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,Dbfj), we analyzed node morphology in homozygous \(\text{Noto}^{\text{Foxj1}}\) embryos by scanning electron microscopy \((n=8)\) and marker gene expression. These analyses showed that nodes in homozygous \(\text{Noto}^{\text{Foxj1}}\) embryos had variable shapes and sizes similar to nodes in \(\text{Noto}^{\text{GFP/GFP}}\) 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 \(\text{Nphp3}\) \((n=4)\) and \(\text{Shh}\) \((n=6)\) expression was variable and compressed, and the width and shape of the crown cell region delineated by expression of \(\text{Dand5}\) \((n=3)\) was no longer horseshoe-shaped but was narrow and irregular (Fig. 4Dc,g,k). By contrast, in \(\text{Foxj1}^{\text{null}}\) mutant embryos \((n=9)\), which retain \(\text{Noto}\) expression (supplementary material Fig. S2), the shape of the node (Fig. 4ch,p), as well as the expression patterns of \(\text{Nphp3}\) \((n=5)\), \(\text{Shh}\) \((n=4)\) and \(\text{Dand5}\) \((n=5)\) (Fig. 4Dd,h,l) were essentially normal, although the nodes appeared somewhat smaller in some \(\text{Foxj1}^{\text{null}}\) mutant embryos.

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**Fig. 3. Foxj1-dependent regulation of Dynlnb2 and Rfx3, but Foxj1-independent regulation of Nphp3 downstream of Noto.** Quantitative RT-PCR analysis of Dynlnb2, Rfx3 and Nphp3 in wild-type \((n=7)\), \(\text{Noto}^{\text{GFP/GFP}}\) \((n=6)\), \(\text{Noto}^{\text{Foxj1/Foxj1}}\) \((n=5)\) and \(\text{Foxj1}^{\text{null}}\) \((n=6)\) LHF embryos. Bars show relative expression levels compared with wild type; error bars indicate s.d. \(p<0.0001\).
Defective polarized localization of nodal cilia in Noto$^{GFP/GFP}$, Noto$^{Foxj1/Foxj1}$ and Foxj1$^{-/-}$ 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$^{GFP/GFP}$ ($n=27/49$ in eight embryos), Noto$^{Foxj1/Foxj1}$ ($n=49/99$ in five embryos) and Foxj1$^{-/-}$ 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. 5Ae-h). Images shown in i and j 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
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 regulates 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 a basal body in a deep indentation of the plasma membrane (Fig. 2Ed). Centriolar structures that were located abnormally deep in the cytoplasm and cilia emerging from such deep basal bodies were also observed in NotoGFP/GFP mutant embryos (Fig. 2Eb) (Beckers et al., 2007) but not in sections from NotoFoxj1/Foxj1 mutant nodes (Fig. 2Ec), 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

cilia and the transcription of genes important for ciliogenesis in mutants lacking Noto function. The rescue of gene expression and cilia structure in NotoFoxj1/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.

Fig. 5. Abnormal positioning of nodal cilia in Noto Foxj1/Foxj1 embryos. (A) (a-d) Representative examples of scanning electron micrographs of node regions of late headfold stage embryos used for evaluation of cilia position. Dashed circles indicate the bases of cilia. (e-h) Representative examples of lateral micrographs of nodes stained by immunofluorescence with antibodies marking basal bodies (γ-tubulin, green) and tight junctions (ZO1, blue) used for evaluation of cilia position. Genotypes are indicated at the top, orientation of embryos is shown at the left. (B) Quantification of cilia position analyzed in electron micrographs (A) and in fluorescent micrographs (B) after antibody staining. (C) Pictures of ciliated cells in the node region of late headfold stage embryos stained with an antibody against DVL2 showing reduced DVL2 levels at the apical edge of the lateral membrane in NotoGFP/GFP and NotoFoxj1/Foxj1 mutants compared with wild type and Foxj1–/–. Scale bars: 4 μm. Genotypes are indicated at the top.
cortex in Foxj1−/− mutant node cells, similar to airway epithelial cells, which might contribute to the abnormal cilia positioning in nodes lacking Foxj1. In NotoGFP/^−^ node cells, 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 Noto/Foxj1 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 Noto/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 Noto/Foxj1 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 Noto/Foxj1 and Noto/Foxj1 mutant (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 Noto/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 Rfx3 function, and has established the regulatory relationship between Foxj1 and Rfx3. Foxj1 is indispensable for functional nodal cilia, and acts upstream of Rfx3. 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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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


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<th>Method used to determine cilia position</th>
<th>Total number of cilia in n embryos</th>
<th>Wild type</th>
<th>Noto\textsuperscript{GFP/GFP}</th>
<th>Noto\textsuperscript{Foxj1/Foxj1}</th>
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<td>Electron microscopy</td>
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<td>103 in four embryos</td>
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