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Notch Signaling Controls the Differentiation of Transporting Epithelia and Multiciliated Cells in the Zebrafish Pronephros

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Notch signaling controls the differentiation of transporting epithelia and multiciliated cells in the zebrafish pronephros

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Epithelial tubules consist of multiple cell types that are specialized for specific aspects of organ function. In the zebrafish pronephros, multiciliated cells (MCCs) are specialized for fluid propulsion, whereas transporting epithelial cells recover filtered-blood solutes. These cell types are distributed in a 'salt-and-pepper' fashion in the pronephros, suggesting that a lateral inhibition mechanism may play a role in their differentiation. We find that the Notch ligand Jagged 2 is expressed in MCCs and that *notch3* is expressed in pronephric epithelial cells. Morpholino knockdown of either *jagged 2* or *notch3*, or mutation in *mind bomb* (in which Notch signaling is impaired), dramatically expands ciliogenic gene expression, whereas ion transporter expression is lost, indicating that pronephric cells are transfated to MCCs. Conversely, ectopic expression of the Notch1a intracellular domain represses MCC differentiation. Gamma-secretase inhibition using DAPT demonstrated a requirement for Notch signaling early in pronephric development, before the pattern of MCC differentiation is apparent. Strikingly, we find that *jagged 2* knockdown generates extra cilia and is sufficient to rescue the kidney cilia mutant *double bubble*. Our results indicate that Jagged 2/Notch signaling modulates the number of multiciliated versus transporting epithelial cells in the pronephros by way of a genetic pathway involving repression of *rfx2*, a key transcriptional regulator of the ciliogenesis program.

KEY WORDS: Pronephros, Jagged 2, Multiciliated cell, Notch3, *double bubble*, *mind bomb*, Zebrafish

INTRODUCTION

The generation of cell diversity during organ development is a crucial event in defining the proportion of specialized cell types that coordinate organ function. How epithelial organ primordia are patterned to give rise to specialized epithelial cells is largely unknown. In the kidney, the nephron is the functional unit of organ physiology. The mature nephron is populated by multiple different cell types that are specialized for blood filtration, and water and ion homeostasis (Seldin and Giebisch, 1992; Vize et al., 2002). Functionally similar cell types in the nephron are typically arranged in discrete tubule segments that process filtered blood sequentially as it passes through the nephron. In other parts of the nephron, functionally distinct cells are intermingled or 'intercalated' between non-like cells. For instance, in the collecting system, intercalated cells that regulate pH homeostasis are morphologically and functionally distinct from adjacent principal cells, whose primary function is water transport (Schuster, 1993; Seldin and Giebisch, 1992). Similar intermingling of functionally distinct cell types is found in other epithelial organs; for example, in the lung, intestine and stomach. In the lung, neuroendocrine cells, which have specialized sensory and secretory functions, are dispersed among airway epithelial cells (Ito et al., 2000). In the intestinal epithelium, mucous-secreting goblet cells are interspersed among villus epithelial cells (Zecchini et al., 2005). In the stomach of the chick, glandular epithelial cells differentiate from an initially homogeneous layer of epithelium and lie interspersed with lumenal epithelial cells (Matsuda et al., 2005). The existence of adjacent non-like cells in these organs suggests that a lateral inhibition mechanism, similar to well-defined mechanisms underlying the development of proneural clusters in *Drosophila* (Artavanis-Tsakonas et al., 1995), may play a role in vertebrate organogenesis. In peripheral neural development in *Drosophila*, clusters of cells destined to become nerves and supporting cells are partitioned by the expression of the Notch ligand Delta in future nerve cells. Delta, acting on Notch receptors in adjacent cells, prevents them from adopting a neural fate (Artavanis-Tsakonas et al., 1995). Using loss- and gain-of-function approaches targeting various elements of vertebrate Notch signaling pathways, lateral inhibition mechanisms have in fact been demonstrated to control the differentiation of neuroendocrine cells in the lung (Ito et al., 2000), goblet cells in the intestine (Crosnier et al., 2005; Zecchini et al., 2005) and gland cells in the stomach (Matsuda et al., 2005).

In Notch-mediated lateral inhibition, a specialized cell type expresses a transmembrane DSL (Delta/Serrate/Lag-2) ligand of Notch which binds a Notch receptor in neighboring cells (Kadesch, 2004). The Notch receptor undergoes regulated intramembrane cleavage to generate an active intracellular domain peptide that transits to the nucleus to participate in transcriptional regulation (Mumm and Kopan, 2000). By binding to and activating the CSL [CBF-1, Suppressor of Hairless (Rbpsuh), Lag-1] transcriptional complex, the Notch intracellular domain (NICD) initiates a transcriptional cascade involving the expression of Hes and HRT/HER/Hey transcriptional repressors (Kadesch, 2004). These, in turn, repress genes that are expressed in Notch-ligand-expressing cells, often including the ligand gene itself, and thereby direct the neighboring cell along a different developmental pathway (Kadesch, 2004). In kidney organogenesis, Notch signaling has been implicated in the development of glomerular vasculature and in segmentation of the nephron (Cheng et al., 2003; McCright, 2003). Expression of the Notch ligand Jagged 1 in endothelial cells and Notch2 in glomerular epithelial cells is required for proper development of the glomerular capillary tuft (McCright et al., 2001). Broadly activating or inhibiting Notch signaling by the expression of activated or dominant-negative Suppressor of hairless proteins have been shown to perturb proximal versus distal nephron fate in *Xenopus* embryos (McLaughlin et al., 2000). Mouse kidney explant

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cultures treated with the gamma-secretase inhibitor DAPT to inhibit Notch cleavage also show a loss of proximal versus distal nephron segments (Cheng et al., 2003). These experiments demonstrate a role for Notch signaling in the specification of relatively large nephron domains. However, there is currently no evidence implicating Notch signaling in the generation of 'salt-and-pepper' patterns of interdigitating cell types within the kidney nephron.

The zebrafish pronephros is an accessible model for nephron cell specification and patterning because it consists of typical vertebrate kidney cell types, and is amenable to both forward and reverse genetic manipulation (Drummond, 2003). Expression of jagged and notch genes in the pronephros suggests that they could play an important role in patterning the pronephric nephron (Zecchin et al., 2005). Evidence for nephron patterning in the pronephros is seen in the segment-specific expression of ion transporters (Nichane et al., 2006; Shmukler et al., 2005), in the expression of receptors and transcription factors in discrete segments (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997; Van Campenhout et al., 2006), and in the disruption of segment-boundary formation in the *pax2.1* (also known as *pax2a* – Zebrafish Information Network) mutant *no isthmus* (Majumdar et al., 2000). In our examination of cilia function in the kidney (Kramer-Zucker et al., 2005) we have found that two types of ciliated cells exist in the pronephros: single ciliated cells that have the morphology of typical transporting epithelial cells, and multiciliated cells that, by several criteria, appear specialized for fluid propulsion in the pronephros. We show here that multiciliated cells are isolated, specialized cells surrounded by non-like transporting epithelia in the distal segment of the pronephros. We present evidence that Notch signaling via Jagged 2-Notch interactions, as well as the downstream transcriptional regulation of ciliogenic genes, is essential in the generation of the 'salt-and-pepper' pattern of multiciliated and transporting epithelial cells in the pronephros. We also demonstrate that transfating cells to the MCC lineage is sufficient to overcome defects in ciliogenesis in a zebrafish cyst mutant and prevent the formation of pronephric cysts.

MATERIALS AND METHODS

Zebrafish lines

Wild-type TL or TÜAB, *double bubble* (m468) and *mind bomb* (m132) zebrafish were maintained and raised as described (Westerfield, 1995). Dechorionated embryos were kept at 28.5°C in E3 solution with or without 0.003% PTU (1-phenyl-2-thiourea, Sigma) to suppress pigmentation and were staged according to somite number (som) or hours post-fertilization (hpf) (Westerfield, 1995). The Na,K-ATPase alpha1A4:GFP transgenic line was created by amplifying an 8112 base-pair (bp) fragment of the Na,K-ATPase alpha1A4-subunit promoter (cDNA accession: NM_131689) from genomic DNA using nested PCR with the following primers:

LNaK-F1: 5′-GGTCGCTGGGCTAAGTCACAGAC-3′
LNaK-F2: 5′-TGGGCTAAGTCACAGACCGCCTA-3′
LNaK-R: 5′-TCCACTCACTCCAAGCCCCATTT-3′
NaKB-R2: 5′-CGCGGATCCCTCACTCCAAGCCCCATTTTCCT-3′.

The 8112 bp fragment was cloned in pGI upstream of EGFP and injected as a circular plasmid into TuAB wild-type embryos at the 1-cell stage. Founders were screened by GFP fluorescence in the pronephros and were out crossed to create a stable transgenic line. For *notch1a* NICD induction, the Tg(*uas:notch1a-intra*) zebrafish line was crossed to the Tg(*hsp70:gal4*) line (Scheer and Campos-Ortega, 1999; Scheer et al., 2002) and heat shocked for 60 minutes at 37°C at either 8, 14 and 28 hpf or at 9, 15 and 29 hpf.

Plasmid clones

Selection of in situ probes was based on information derived from the high-throughput in situ screen carried out by Thisse et al. (ZFIN gene expression data, http://zfin.org). Plasmid probes were ordered from either Open Biosystems or from the Zebrafish International Resource Center (ZIRC): *shippo1* (BC054587); *rfx2* (B1325076); *trpM7* (NM_001030061); *slc13a1* (NM_199281); *her9* (AF301264). *jagged 2*, NM_131862 and *jagged 1b*, NM_131863 (previously *jagged 3*) were gifts from A. Chitnis (Laboratory of Molecular Genetics, NIH/NICHD, USA), and the zebrafish *notch3*, NM_131549 was a gift from B. Weinstein (Laboratory of Molecular Genetics, NIH/NICHD, USA). The *fleer* (*flr*) cDNA encodes a TPR protein (N.P. and I.A.D., unpublished).

In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Thisse and Thisse, 1998). For two-color in situ hybridization, probes were synthesized using DIG-UTP or FITC-UTP. Stained embryos were cleared in Benzylbenzoate:Benzyl alcohol and photographed on a Lietz MZ12 or Nikon E800 microscope equipped with a Spot Image digital camera. Two-color stained embryos were cleared in glycerol and photographed after removal of the yolk extension. Two-color fluorescent in situ hybridization (S. Holley, Yale University, New Haven, CT, personal communication) was performed using DIG-UTP and FITC-UTP-labeled RNA probes followed by sequential detection using HRP labeled anti-DIG and HRP-labeled anti-FITC antibodies (Boehringer Mannheim) and fluorescent tyramide amplification. Following RNA-probe hybridization, embryos were blocked in 150 mM maleic acid, 100 mM NaCl pH 7.5 with 2% Boehringer blocking agent, and were then incubated with HRP anti-FITC at 1:500 dilution for 4 hours in blocking solution. After washing, anti-FITC antibody was visualized with TSA Plus Fluorescein Solution (Perkin Elmer) for 45 minutes, dehydrated in methanol and quenched with 1% H_2O_2 in methanol for 30 minutes. After rehydrating, embryos were blocked again and incubated with HRP anti-DIG at 1:1000 dilution for 4 hours, then washed and reacted with TSA Plus Cy3 solution (Perkin Elmer) for 45 minutes. For combined fluorescent *shippo1* DIG in situ/anti-acetylated tubulin staining, embryos were incubated with both anti-acetylated tubulin (clone 6-11B-1; Sigma) and HRP anti-DIG (Boehringer Mannheim) after hybridization. After complete fluorescent in situ hybridization, embryos were refixed with 4% PFA, and were then washed and blocked in 150 mM maleic acid, 100 mM NaCl pH 7.5 with 10% NGS for 2 hours at room temperature. Acetylated tubulin was visualized with Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes) in blocking solution. Embryos were cleared in glycerol, mounted and viewed on a Zeiss LSM5 PASCAL confocal fluorescence microscope.

Morpholino antisense oligonucleotides

Morpholino antisense oligonucleotides (Gene Tools, Philomath, OR) were designed to target either the translation start or an exon splice-donor site causing splicing defects of the mRNA. The following morpholinos were used:

j2exon 20: 5′-TCTTTGAGATACTCACTGATACGGC-3′
j2exon 20 control: 5′-CGGCATAGTCACTCATAGAGTTTCT-3′
rfx2exon 6: 5′-GGGTGTAGTCTGACCTGGTAC-3′
rfx2exon 6 control: 5′-CATGGTCCAGTCTGATGTGGG-3′
notch3exon 27: 5′-TGACCAACTCACTTCATGCCCAGTG-3′
jagged 2 ATG: 5′-TCCTGATACAATTCCACATGCCGCC-3′
jagged 1b(3) ATG: 5′-CTGAACTCCGTCGCAGAATCATGCC-3′.

Morpholino oligos were diluted in 100 mM KCl, 10 mM HEPES, 0.1% phenol red (Sigma) and injections were performed using a nanoliter2000 microinjector (World Precision Instruments). Injection concentrations were 0.2-0.5 mM and injection volume was 4.6 nl/embryo (7-16 ng morpholino/embryo). The molecular defect caused by splice-donor morpholinos was verified by reverse-transcriptase PCR from total RNA from a single morphant embryo with nested primers in flanking exons.

Histochemistry and immunohistochemistry

For histology, embryos were fixed with 4% formaldehyde in PBS at 4°C overnight followed by dehydration and embedding in JB-4 (Polysciences), and were then sectioned at 5-7 μm. Slides were stained in methylene blue/azure II (Humphrey and Pittman, 1974) and examined using a Nikon E800 microscope. For acetylated tubulin staining, the embryos were fixed in Dent's Fix (80% methanol/20% DMSO) at 4°C overnight. After rehydration they were washed in PBS plus 0.5% Tween20 and blocked in PBS-DBT (PBS plus 1% DMSO, 1% BSA and 0.5% Tween20) with 10%

normal goat serum (NGS) (Sigma) at room temperature for 2 hours. Primary-antibody incubation in PBS-DBT 10% NGS [1:500 monoclonal anti-acetylated tubulin 6-11B-1 (Piperno and Fuller, 1985), Sigma] was at 4°C overnight. The embryos were washed in PBS/0.5% Tween20 and blocked in PBS-DBT 10% NGS at room temperature for 1 hour, and were then incubated in 1:1000 goat anti-mouse Alexa Fluor 546 (Molecular Probes) in PBS-DBT 10% NGS at 4°C overnight. After rinsing in PBS, the embryos were washed with methanol, equilibrated in clearing solution (1:2 benzoyl-alcohol:benzoyl-benzoate) and examined using a Zeiss LSM5 Pascal confocal microscope. For combined *jagged 2* DIG in situ/anti-acetylated-tubulin staining, embryos were incubated with both anti-acetylated tubulin 6-11B-1 and anti-DIG after hybridization. Acetylated tubulin was visualized with diaminobenzidine histochemistry using a HRP ABC kit (Vector Laboratories) before being embedded and sectioned. For analysis of GFP expression in tissue sections, GFP-transgenic embryos were stained with polyclonal anti-rabbit GFP antibody (G1544, Sigma) in whole mount at 1:100 followed by an Alexa Fluor 488 (Molecular Probes) goat anti-rabbit secondary antibody at 1:500.

Electron microscopy

Embryos were prepared for electron microscopy by previously published protocols (Drummond et al., 1998).

High-speed video microscopy

PTU-treated embryos were put in E3 egg water containing 40 mM 2,3-butanedione monoxime (BDM, Sigma) for 5 minutes to stop the heart beat and were then changed to 20 mM BDM, which contained egg water, for observation. The embryos were then analyzed using a 40×/0.55 water-immersion lens on a Zeiss Axioplan microscope (Zeiss, Germany) equipped with a high-speed Photron FastCAM-PCI 500 video camera (Photron). Images of beating cilia were acquired at 250 frames per second using Photron FastCAM version 1.2.0.7 (Photron Ltd). Image processing was done using Photoshop 7.0 (Adobe) and movies compiled in Graphic Converter version 4.5.2 (Lemke Software, Germany) and Quicktime (Apple).

DAPT treatments

DAPT was used at a final concentration of 100 μM diluted in embryo medium from a 10 mM stock in DMSO (Geling et al., 2002). Zebrafish embryos treated with DAPT were kept at 28°C until analysis at 34 hpf. Control embryos were mock treated with embryo medium containing the same concentration of DMSO carrier only.

RESULTS

Multiciliated cells and transporting epithelial cells are distinct, specialized cell types in the pronephros

We have shown previously that motile cilia in the zebrafish pronephros are required for the normal high rate of fluid output from this organ and that defects in cilia function are sufficient to cause cyst formation in the proximal pronephric tubules (Kramer-Zucker et al., 2005). More-detailed examination of ciliated cells in the pronephros showed that the majority of cells possessed a single cilium, while a subset of cells were multiciliated and extended up to 20 cilia from their apical surface into the pronephric lumen (Fig. 1). Acetylated tubulin immunofluorescence of whole-mount zebrafish larvae at 52 hpf revealed bright bundles of cilia in the pronephric lumen, which were interspersed among cells with a single cilia (Fig. 1A,B). Ultrastructurally, these bundles of cilia originated from individual cells characterized by multiple apical basal bodies and apically oriented mitochondria (Fig. 1C-F). By contrast, the surrounding single-ciliated cells possessed a microvillar brush border and basolaterally oriented mitochondria, a morphology typical of transporting epithelia. The distribution of multiciliated cells (MCCs) as isolated individual cells along the pronephros was also apparent in high-speed micro video images of the pronephros (see Movie 1 in the supplementary material). Bundles of cilia emanating from MCCs beated in a coordinated wave pattern, in

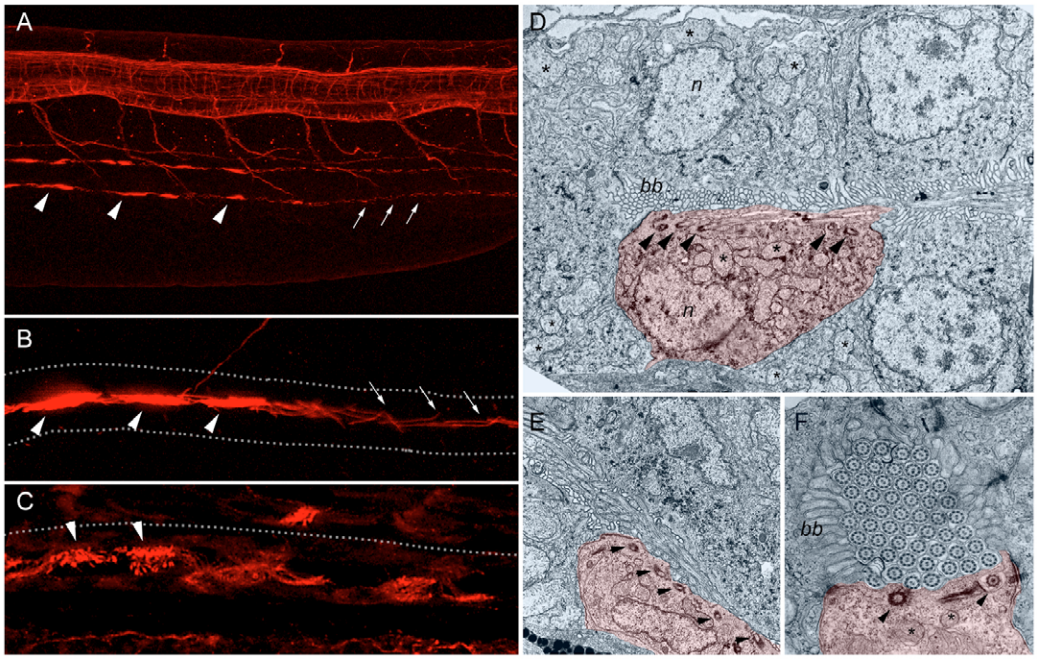

Fig. 1. Distribution of multiciliated cells and transporting epithelia containing single cilia in the pronephros. (**A**) Whole-mount immunofluorescence of the trunk region of a 48 hpf embryo stained with anti-acetylated tubulin reveals bright cilia bundles (arrowheads) in the pronephric lumens, as well as single ciliated cells (arrows) in a more caudal nephron segment. (**B**) At higher magnification, compressed bundles of cilia (arrowheads) and single cilia (arrows) can be observed in the pronephric lumen. (**C**) Bundles of cilia emanating from individual cells project into a distended lumen of a mechanically obstructed pronephros. Dotted lines in B and C outline the pronephric tubules. (**D-F**) Electron micrographs of the pronephros show isolated multiciliated cells (MCC; false-colored in red) interspersed among transporting epithelial cells (false-colored in light blue). Arrowheads show apical basal bodies. (D) MCCs are distinguished by multiple apical basal bodies, by the lack of a brush border (*bb*), by a small basal cell surface and by multiple apical cilia (asterisks). *n*, nucleus. (E) Example of an MCC with multiple apical cilia basal bodies, and bundles of cilia in the lumen. (F) Cross section of a pronephric tubule in a 7 dpf larva, showing a single MCC with multiple basal bodies, apical mitochondria (asterisks) and bundles of cilia fitting tightly into the pronephric lumen.

contrast to the single cilia on neighboring transporting epithelial cells, which appeared to beat autonomously (see Movie 1 in the supplementary material).

Consistent with the abundance of cilia on MCCs, we found that genes for axonemal structural proteins and regulators of cilia formation were highly expressed in a pattern similar to MCCs in the pronephros (Fig. 2). *shippo1* (*zgc:63985* – Zebrafish Information Network) encodes an axonemal protein originally isolated from male germ cells (Egydio de Carvalho et al., 2002) (Fig. 2A) and *rfx2* belongs to the family of X-box-binding transcription factors, which act as master regulators of the ciliogenesis program (Bonnafe et al., 2004; Efimenko et al., 2005; Swoboda et al., 2000). *rfx2* was initially expressed throughout the pronephros, implying a role for this transcription factor in the development of both single- and multi-ciliated cells (data not shown). By 34 hpf, however, *rfx2* was concentrated in a spotted pattern similar to the distribution of multiciliated cells (Fig. 2B). *shippo1*- and *rfx2*-positive cells are present throughout the distal segment of the pronephric nephron (Nichane et al., 2006) but were not present in the most-caudal nephron segment (Fig. 2A,B). Because cells directly adjacent to MCCs appear by electron microscopy to be more typical of transporting epithelia (Fig. 1D-F), we examined ion channel gene expression in similar regions of the pronephros. Cells in the 'early distal' segment of the pronephric nephron expressed the cation transporter *trpM7* (Fig. 2C) and the sodium-sulfate co-transporter *slc13a1* (Fig. 2D). Whereas epithelial cells expressing these two channel genes appeared to coexist with *shippo1*- and *rfx2*-positive cells in the early distal segment, the distribution of *shippo1*- and *rfx2*-positive cells further extends distally into the 'late distal' segment of the pronephros (Nichane et al., 2006).

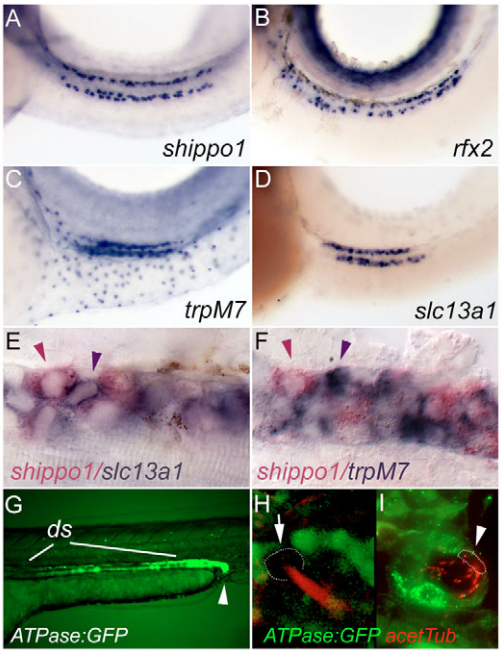

Fig. 2. MCCs and transporting epithelia co-exist as separate cell types in the early distal segment of the pronephric nephron. (**A**) Expression of the axonemal-sheath gene *shippo1* in the pronephros of a 34 hpf embryo. (**B**) Expression of the transcription factor *rfx2* in individual pronephric cells. (**C**) Expression of the cation transporter *trpM7* in the early distal segment of the pronephric nephron. (**D**) Expression of the sodium-sulfate co-transporter *slc13a1* in the early distal segment. (**E**) Double in situ hybridization of the MCC marker *shippo1* (red) and *slc13a1* (purple) in distinct but adjacent cells of the early distal segment in a 34 hpf embryo. (**F**) Double in situ hybridization of *shippo1* and *trpM7* in early distal segment cells. (**G**) Expression of GFP from the Na,K-ATPase alpha a1A4 subunit promoter is uniform in the most caudal nephron segment (arrowhead) and heterogeneous in the distal nephron. (**H**,**I**) Double staining for GFP and acetylated tubulin (red) in confocal z-series projections (H) and in histological sections (I) shows that the Na,K-ATPase alpha a1A4 promoter is not active in MCCs (arrows, arrowhead; cell bodies are outlined with dashed line).

To confirm that *shippo1/rfx2* and *trpM7/slc13a1* marked non-equivalent, but adjacent, cell types in the pronephros, we performed two-color whole-mount in situ hybridization and double fluorescent in situ hybridization with these probes. Two-color whole-mount in situ hybridization verified that *trpM7/slc13a1*-expressing transporting epithelial cells and *shippo1*-expressing cells are indeed two distinct cell types that exist side by side in the pronephros (Fig. 2E,F). To further distinguish MCCs from transporting epithelia, we created a transgenic line of zebrafish that expresses GFP from the Na,K-ATPase alpha1A4 gene promoter. This Na,K-ATPase subunit is highly expressed in the pronephros, where, by analogy to mammalian kidney epithelia, it is likely to power a variety of ion-transport activities (Seldin and Giebisch, 1992). In the distal segment of the pronephros, the Na,K-ATPase:GFP transgene was expressed in a subset of cells (Fig. 2G) and, based on co-staining for cilia bundles with anti-acetylated tubulin, was excluded from MCCs (Fig. 2H,I). The complementary expression pattern of axonemal genes/cilia bundles with genes for ion transporters suggests that MCCs and transporting epithelia are distinctly different cell types, intermixed in the distal segment of the pronephros.

Using a combination of *shippo1* fluorescent in situ hybridization and anti-acetylated tubulin immunofluorescence, we found that *shippo1*-positive cells were multiciliated (Fig. 3A,B). Neighboring cells with a single cilia were *shippo1*-negative (Fig. 3A,B). Using *shippo1* as a reference probe for MCCs in double fluorescent in situ hybridization with ciliogenic or ion channel gene probes, we confirmed that other ciliogenic genes – including *fleer* (*flr*), a tetratricopeptide-repeat protein required for ciliogenesis (N.P. and I.A.D., unpublished) (Fig. 3C-E), and *rfx2* (Fig. 3F-H) – were co-expressed with *shippo1* in MCCs. Conversely, the ion channel genes for *slc13a1* (Fig. 3I-K) and *trpM7* (Fig. 3L-N) were expressed in a non-overlapping, adjacent set of cells in the pronephros.

The Notch ligand Jagged 2 is expressed in multiciliated cells

The apposition of different epithelial cell types in the pronephros suggested that a lateral inhibition mechanism, possibly involving Notch signaling, might play a role in pronephric cell patterning. Because the Notch receptor *notch3* and the Notch ligands *jagged 2* and *jagged 1b* (previously *jagged 3*) have recently been reported to be expressed in the pronephros (Lawson et al., 2001; Zecchin et al., 2005), we examined the expression of Notch signaling genes in relation to MCCs and transporting epithelial cells. Interestingly, *jagged 2* was expressed in the pronephric mesoderm during early somitogenesis (Fig. 4A) and was later expressed in a subset of cells in the pronephros in a pattern similar to that of the MCC-marker gene expression (Fig. 4B). Double staining by *jagged 2* in situ hybridization and anti-acetylated tubulin immunohistochemistry demonstrated that *jagged 2*-positive cells were indeed multiciliated (Fig. 4C). A bundle of cilia could be seen originating from a single *jagged 2*-expressing cell in a cross section of the pronephros (Fig. 4C). To confirm further the identity of *jagged 2*-expressing cells, we performed two-color and double fluorescent in situ hybridization

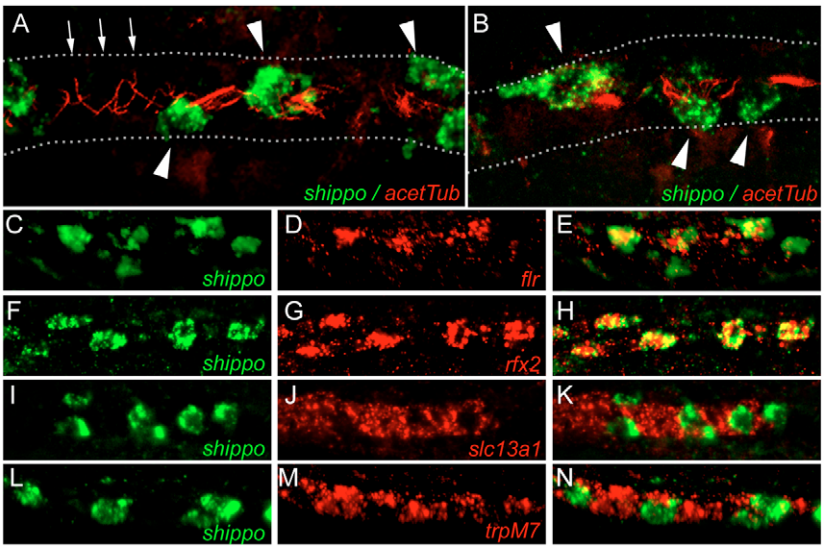

Fig. 3. Confocal fluorescent in situ hybridization analysis of ion transporter and ciliogenic gene expression in the pronephros. (**A**,**B**) Double staining for *shippo1* mRNA (in situ hybridization; green) and acetylated tubulin (immunofluorescence; red) in confocal projections reveals several examples of cilia bundles emanating from *shippo1*-positive cells (arrowheads) into the lumen; single cilia emanate from *shippo1*-negative cells (small arrows). Dotted lines outline the pronephros; posterior is to the right. (**C-E**) Co-expression of *shippo1* (C; green) and *fleer* (D; red) in single cells of the pronephric tubules. (E) Merged image. (**F-H**) Co-expression of *shippo1* (F; green) and *rfx2* (G; red) in single cells of the pronephric tubules. (H) Merged image. (**I-K**) Expression of *shippo1* (I; green) and *slc13a1* (J; red) in distinct but adjacent cells of the pronephric tubules. (K) Merged image. (**L-N**) Expression of *shippo1* (L; green) and *trpM7* (M; red) in distinct but adjacent cells of the pronephros. (N) Merged image.

with *jagged 2* and *shippo1* probes. By both methods, we found that *shippo1* was co-expressed with *jagged 2* in a distinct subset of cells (Fig. 4D,E-G), confirming that *jagged 2* is specifically expressed in MCCs.

Jagged 2 represses MCC fate

Expression of *jagged 2* mRNA in MCCs suggested that it could be required in some way for MCC cell differentiation or, by analogy to Notch signaling in other contexts, it could give rise to the intercalated pattern of MCCs by repressing the multiciliated cell fate in neighboring cells. To distinguish between these possibilities, we disrupted the expression of *jagged 2* with antisense morpholino oligos that targeted either the *jagged 2* ATG initiation codon (J2atgMO; protein knockdown) or the *jagged 2* exon 20 splice donor (J2ex20MO; mRNA mis-splicing). The J2atgMO has previously been shown to block *jagged 2* protein translation (Lorent et al., 2004) and the J2ex20MO was found to delete exon 20 and generate an in-frame stop codon immediately following the coding sequence of exon 19, with the resultant mRNA predicted to encode a truncated *jagged 2* extracellular domain peptide (see Fig. S1 in the supplementary material). Injection of either morpholino oligo gave rise to the same phenotype: the number of MCCs was dramatically expanded in the pronephros (Fig. 5A-I). Injection of a control invert sense morpholino had no effect on the expression pattern of the MCC markers *shippo1*, *flr* or *rfx2* (Fig. 5). Sections of whole-mount stained embryos confirmed that the single-cell pattern of MCC-marker expression (Fig. 5J) expanded to include all tubule cells in the section in *jagged 2* morphants (Fig. 5K,L). Interestingly, expression of *jagged 2* itself was also dramatically expanded in *jagged 2* morphants (Fig. 5R,U).

One interpretation of the enhanced expression of MCC markers in *jagged 2* morphants would be that MCC cell number was increased by the transfating of transporting epithelial cells to MCCs. Alternatively, it is possible that MCC and transporting-cell phenotypes could co-exist in the same cells and that *jagged 2* loss of function may simply be derepressing axonemal gene expression. We therefore examined *jagged 2* morphants for expression of the transporters *trpM7*, *slc13a1* and Na,K-ATPase alpha1A4:GFP. Strikingly, *jagged 2* morphants showed a complete loss of all mid-segment ion-transporter gene expression (Fig. 5M-Q) as well as a segment-specific reduction in Na,K-ATPase alpha1A4:GFP expression (Fig. 5S,T). As a further test for specificity, we also knocked down *jagged 1b* expression and assessed MCC and transporter gene expression. *jagged 1b* knockdown had no effect on marker gene expression, indicating that the observed effects were specific to *jagged 2* (data not shown). The results suggest that *jagged 2* acts as a repressor of a genetic pathway leading to MCC differentiation and that part of this patterning mechanism involves

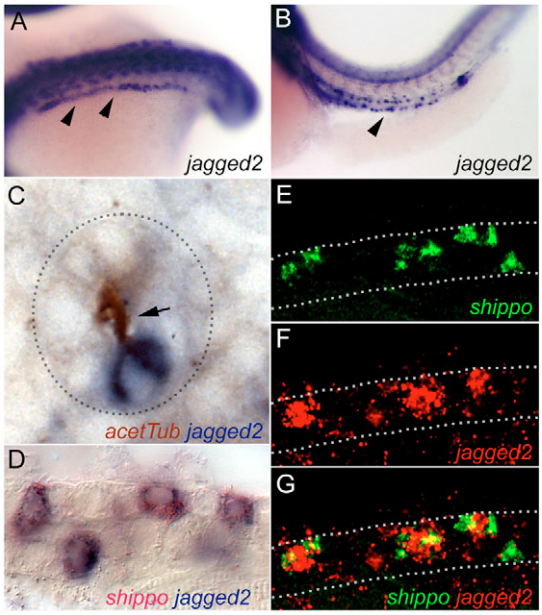

Fig. 4. *jagged 2* expression in multiciliated cells. (**A**,**B**) Whole-mount in situ hybridization showing expression of *jagged 2* (arrowheads) at the 18-somite stage in cells of the intermediate mesoderm (A) and in individual cells of the pronephros at 42 hpf (B). (**C**) Cross section of the pronephros double stained for acetylated tubulin (HRP:DAB immunohistochemistry) and *jagged 2* (in situ hybridization) shows that isolated *jagged 2*-positive cells possess apical cilia bundles (arrow). Dashed line outlines the cross section of the pronephric tubule. (**D**) Double in situ hybridization with the MCC marker *shippo1* (red) and *jagged 2* (blue) demonstrates *jagged 2*/*shippo1* co-expression in isolated single cells. (**E-G**) Confocal optical section of the pronephros in situ hybridized for *shippo1* mRNA (E; green) and *jagged 2* (F; red) confirm *jagged 2* expression in *shippo1*-positive MCCs. (G) Merged image. Dotted lines outline the pronephros.

jagged 2 repression of its own mRNA synthesis in a subset of pronephric cells. Additionally, the fates of MCCs and transporting epithelial cells appear to be mutually exclusive, with *jagged 2* repression of MCC fate being necessary to allow neighboring cells to acquire a secondary, transporting-epithelial-cell fate.

A role for Notch3 in mediating Jagged 2 signaling in the pronephros

Notch3 has previously been shown to be expressed in the pronephros (Lawson et al., 2001) and was a reasonable candidate for mediating the effects of Jagged 2. We confirmed that *notch3* mRNA was expressed strongly in the proximal and distal pronephros, and is diffusely expressed in the pronephric midsegment (Fig. 6A,B). Knockdown of *notch3* with a morpholino oligo targeting *notch3* exon 27 produced a morphant 'allele' truncated after exon 26 (stop codon following 20 mis-sense amino acids; see Fig. S1 in the supplementary material) and lacking both the transmembrane domain and the *notch3* intracellular domain required for nuclear signaling. Similar to *jagged 2* morphants, both *shippo1*-positive (Fig. 6C,D) and *flr*-positive (Fig. 6E,F) cells were significantly increased in number in *notch3* morphants, whereas the expression of *slc13a1* (Fig. 5G,H) and *trpM7* (Fig. 5I,J) was nearly completely abolished in the early-distal pronephros.

Regulation of pronephric cell patterning requires *mind bomb* activity

mind bomb mutants lack an E3 ubiquitin ligase that is required to process Notch ligands and to facilitate Notch signaling (Itoh et al., 2003). To test whether Jagged 2 signaling required *mind bomb* activity, we examined MCC and transporting-cell markers in *mind bomb* homozygotes. Consistent with the *jagged 2*- and *notch3*-morphant phenotypes, expression of the MCC markers *shippo1* and *jagged 2* was dramatically expanded in *mind bomb* homozygotes (Fig. 7A-D). *shippo1* expression in single cells of the distal segment (Fig. 7A) was expanded to include nearly all cells of this segment (Fig. 7B, *ds*). In addition, the number of *shippo1*-positive cells was significantly increased in the proximal segment of the pronephros (Fig. 7B, *ps*). Expression of the transporting-cell markers *trpM7* and *slc13a1* was nearly completely abolished (Fig. 7E-H). These results indicate that *mind bomb*-processing activity is required for *jagged 2*/*notch3* patterning of the pronephros.

Timing of Jagged 2/Notch3 signaling during pronephric development

jagged 2 mRNA expression is initiated early in the intermediate mesoderm and is maintained in fully differentiated MCCs (Fig. 4A,B). To determine when Jagged 2/Notch3 signaling acts to restrict the MCC fate and to assess whether continued Jagged 2/Notch3 signaling is required to generate the mature pattern of cell types in the pronephros, we used the gamma-secretase inhibitor DAPT to block notch cleavage (Geling et al., 2002) at different time points during pronephric development. Expansion of MCC fate (increased *shippo1* expression; Fig. 8A-C) and the loss of *trpM7*-expressing transporter cells (Fig. 8G-I) occurred when DAPT was added to egg water at 9.5 hpf, but not if it was added later, at 24 hpf (Fig. 8). Interestingly, *slc13a1* expression was sensitive to inhibition of Notch signaling at later developmental stages (24+ hpf; Fig. 8J-L), indicating that, although expressed in the same cells, *trpM7* and *slc13a1* may respond to Notch signaling in different ways. The effect of DAPT on MCC differentiation suggested that cell fate choice is decided during late gastrulation and early somitogenesis, and that continued Notch3 signaling after 24 hpf may not be required

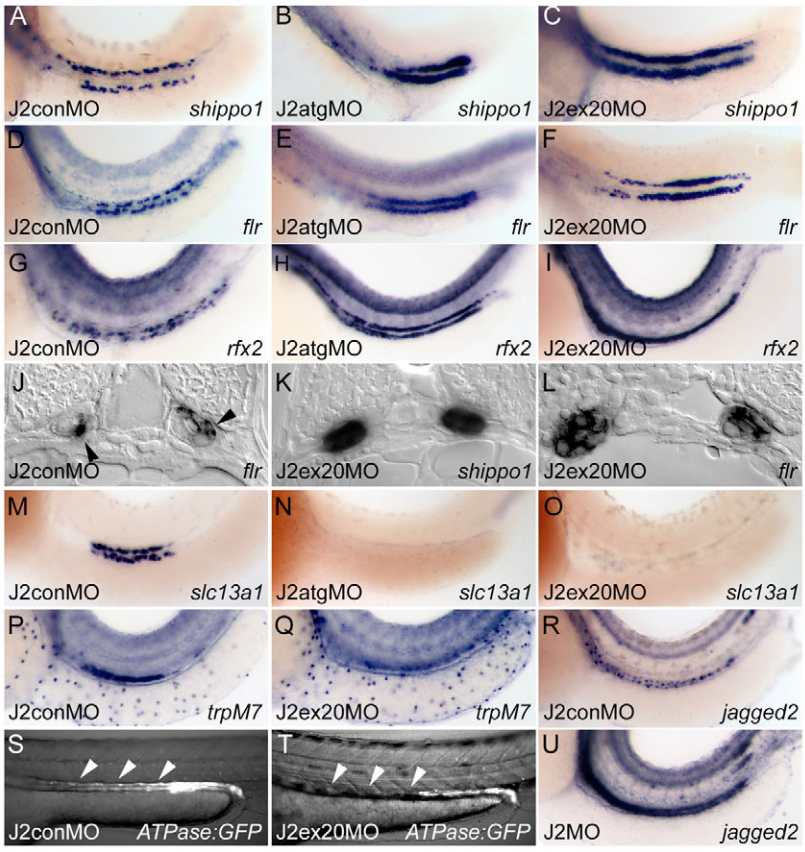

Fig. 5. *jagged 2* loss of function transfates pronephric cells to MCCs. Three markers of MCCs – *shippo1* (**A-C**), *flr* (**D-F**) and *rfx2* (**G-I**) – show expanded expression in the pronephros of *jagged 2* morphants at 34 hpf. Control invert morpholino (A,D,G) had no effect on MCC-marker expression, whereas ATG blocking (J2atgMO; B,E,H) and exon 20 mis-splicing morpholinos (J2ex20MO; C,F,I) resulted in uniform expression of MCC markers. (**J**) MCCs exist as single cells (arrowheads; *flr*-expressing cells) in the wild-type pronephros in cross section. (**K**,**L**) Expressions of *shippo1* (K) and *flr* (L) expand to include all cells in tubule cross sections in J2exon20 morphant embryos. (**M-O**) *slc13a1* expression seen in the control (M) is lost in J2atg (N) and J2exon20 (O) morphants. Similarly, the observed expression of *trpM7* in control morpholino-injected embryos (**P**) is lost in J2exon20 morphants (**Q**). Control *jagged 2* morpholino does not alter the single-cell *jagged 2* mRNA expression pattern (**R**), whereas combined injection of both *jagged 2* atg and exon 20 antisense morpholinos (**U**) result in expanded *jagged 2* mRNA expression. (**S**) Na,K-ATPase:GFP transgenic zebrafish express GFP in the pronephros distal segments (arrowheads). (**T**) GFP expression is lost specifically in the distal segment of *jagged 2* exon 20 morphant embryos (arrowheads).

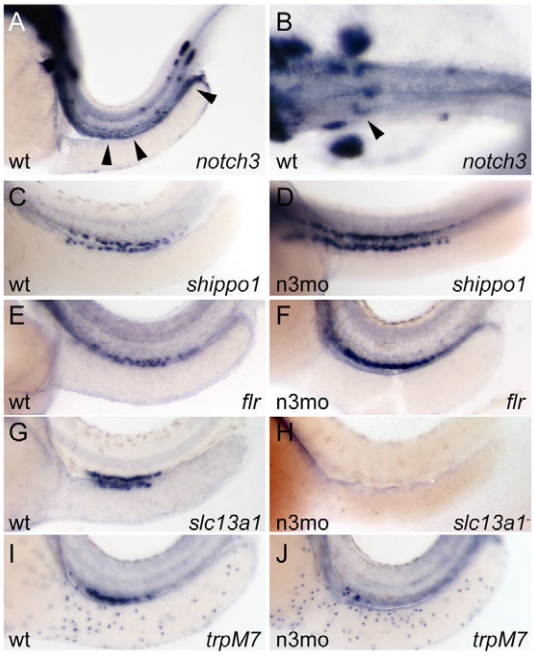

Fig. 6. Notch3 mediates Jagged 2 signaling in the pronephros. (**A**) Expression of *notch3* in the pronephros (arrowheads). (**B**) Expression of *notch3* in the proximal pronephric tubules (arrowhead). (**C**,**D**) *shippo1* expression in single cells of control embryos at 34 hpf (C) is expanded by *notch3* exon 27-morpholino injection (D). (**E**,**F**) Similarly, expression of *flr* that is observed in control embryos (E) is expanded in *notch3* exon 27 morphants (F). (**G**,**I**) Transporting-cell markers *slc13a1* (G) and *trpM7* (I) are greatly reduced in *notch3* exon 27 morphants (**H** and **J**, respectively).

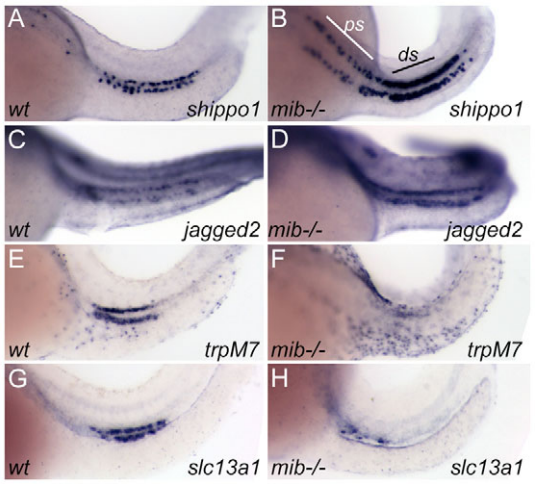

Fig. 7. Transfating of pronephric cells in the *mind bomb* mutant. (**A**,**B**) *shippo1* expression in control wild-type embryos is restricted to single cells (A), whereas, in *mind bomb* homozygotes (B), pronephric distal segment cells (*ds*) are uniformly positive and there is an increase in *shippo1*-positive cells in the proximal segment (*ps*). (**C**,**D**) Wild-type *jagged 2* expression (C) is also expanded in *mind bomb* homozygotes (D). (**E**,**G**) *trpM7* (E) and *slc13a1* (G) expression is almost completely lost in *mind bomb* homozygotes (**F** and **H**, respectively).

to limit the number of MCCs. To further refine the time window in which Jagged 2/Notch signaling acts, we treated embryos with DAPT starting at progressively later time points and assayed the number of MCCs at 34 hpf by counting the number of *shippo1*-positive cells in each pronephric nephron. As shown in Fig. 9A, control pronephric tubules contained, on average, 26±4 (±s.d., n=17) MCCs, and treatment with 100 μM DAPT at 9.5 hpf increased this number to 67±14 (n=8; although this is possibly an underestimate of MCC number given the difficulty in counting overlapping *shippo1*-positive cells). MCC differentiation was sensitive to DAPT only during a narrow time window (around 9-10 hpf), and later treatment at 12 or 15 hpf did not significantly affect the number of MCCs (Fig. 9A).

Ectopic expression of the Notch1a intracellular domain blocks MCC differentiation

Gamma-secretase cleavage of Notch proteins liberates the NICD, which is then transported to the nucleus where it acts as a transcriptional regulator. Our results on Jagged/Notch loss of function indicate that Notch signaling acts as a repressor of the MCC fate. To directly test this, we expressed the Notch1a ICD under heat-shock control and assayed *shippo1* expression in the pronephros. Heat-shock induction of the NICD at 8 hpf dramatically reduced the number of *shippo1*-positive MCCs at 34 hpf (Fig. 8E); when the NICD was induced by heat shock at 9 hpf (Fig. 8F), MCC number was also reduced, but to a lesser extent. *shippo1*-positive cells were counted in each case and the results quantified (Fig. 9B). Control heat-shocked siblings had 27±2 (±s.d.; n=10) MCCs per tubule, whereas those in which the NICD was induced at 8 hpf and 9 hpf had 9±3 (±s.d.; n=35) and 17±4 (±s.d.; n=7) MCCs per tubule, respectively. These results confirm that Notch signaling acts as a repressor of MCC fate early in the development of the pronephros.

Enhanced ciliogenesis and rescue of the *double bubble* kidney cyst mutant by *jagged 2* knockdown

The strong expression of ciliogenic genes and the absence of transporter gene expression suggested that loss of Jagged 2/Notch signaling could cause cells to fully transfate to the MCC cell type. To assess whether *jagged 2*-morphant pronephric cells acquired the functional character of MCCs, we examined the abundance and motility of cilia in morphant embryos. Immunostaining with anti-acetylated tubulin revealed that the loss of *jagged 2* function increased the abundance of lumenal cilia in the pronephros (Fig. 10A,B). Moreover, the newly formed cilia in *jagged 2*-morphant pronephroi all beated rapidly (see Movie 2 in the supplementary material), similar to the behavior of wild-type pronephric cilia (see Movie 1 in the supplementary material). To better visualize beating cilia, the pronephric opening of these embryos was mechanically obstructed to expand the pronephric lumen. Notch3 morphants also showed an increase in cilia bundles in the pronephros (data not shown). These results indicate that loss of Jagged 2/Notch signaling not only enhanced axonemal gene expression, but was sufficient to transfate pronephric cells to fully functional MCCs.

Motile cilia in the pronephros are required to maintain high rates of fluid output and prevent backpressure, which can lead to pronephric cyst formation (Kramer-Zucker et al., 2005). The transfating of epithelial cells to MCCs in *jagged 2* morphants raised the possibility that the enhancement of the ciliogenic program might be sufficient to compensate for deficiencies in ciliogenesis that are observed in a class of pronephric cyst mutants. We therefore tested whether *jagged 2* knockdown in pronephric cyst mutant

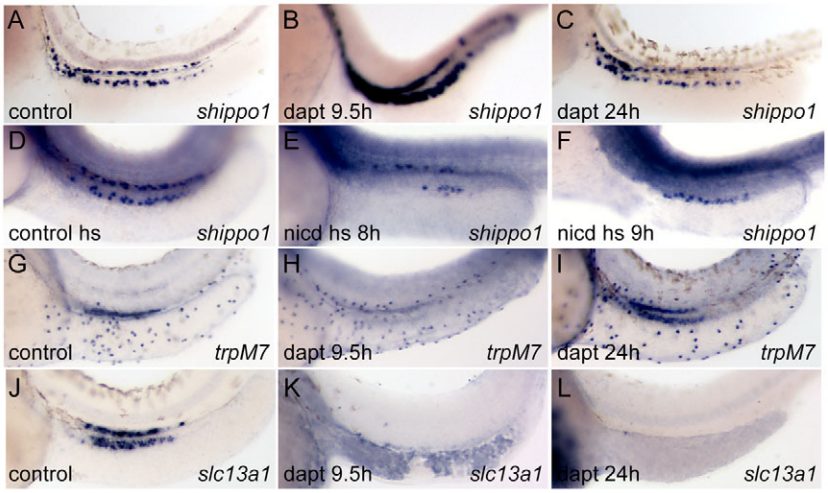

Fig. 8. Inhibition of gamma-secretase and the timing of Notch signaling in pronephric development. All whole-mount in situ hybridization was performed at 34 hpf. (**A-C**) The wild-type pattern of *shippo1* expression (A) is dramatically expanded when gamma-secretase is inhibited with 100 μM DAPT starting at 9.5 hpf (B) but not when DAPT is added later, at 24 hpf (C). (**D-F**) Conversely, the number of *shippo1*-positive cells seen in control embryos (D) is dramatically reduced in the pronephros of embryos induced to ectopically express the Notch1a intracellular domain under the control of HS-GAL4 induction (nicd hs) at either 8 hpf (E) or 9 hpf (F). (**G-I**) *trpM7* expression is lost when DAPT is added at 9.5 hpf (H), but not when it is added later, at 24 hpf (I). (**J-L**) *slc13a1* expression is lost when DAPT is added at 9.5 hpf (K), and also when DAPT is added later, at 24 hpf (L).

embryos would have any effect on cilia structure, cilia motility and/or pronephric cyst formation. We screened five different pronephric cyst mutants [*flr*, *oval* (*ift88*), *tg238a*, *schmalhans* and *double bubble*] for potentially beneficial effects of *jagged 2*-morpholino injection on ciliogenesis. Based on an apparent improvement in phenotype (absence of cysts and edema), one of these mutants, *double bubble* (m468) (Drummond et al., 1998), was chosen for further analysis. The *double bubble (dbb)* mutant is characterized by a loss of cilia structure (Fig. 10E,G) with consequent lumen dilatation and pronephric cyst formation (Drummond et al., 1998). *dbb* homozygotes can also be identified by a characteristic ventral axis curvature (Drummond et al., 1998). High-speed micro videos showed that *dbb* homozygotes lack detectable motile cilia (see Movie 4 in the supplementary material) in comparison to similar pronephric segments in wild-type embryos (see Movie 3 in the supplementary material). Significantly, *jagged 2* knockdown rescued normal cilia formation in *double bubble*-mutant embryos (Fig. 10C,D). Rescued cilia bundles in *dbb* homozygotes were actively motile (see Movie 5 in the supplementary material), similar to wild-type cilia (see Movie 3 in the supplementary material). Remarkably, *jagged 2* knockdown completely prevented pronephric cyst formation in *dbb* homozygotes and restored wild-type pronephric structure to mutant embryos (Fig. 10E-H). In three separate experiments, injection of *jagged 2* exon 20 donor-morpholino into embryos from pair matings of *dbb* heterozygotes prevented cyst formation (embryos with cysts/total embryos in clutch: 0/42, 0/46 and 0/41). Control morpholino-injected embryos developed cysts at standard Mendelian frequencies (25%) and injection of the same concentration of *jagged 1b* morpholino or control morpholinos had no effect on the *dbb*-mutant phenotype (data not shown). We also tested whether DAPT treatments that significantly increased MCC marker gene expression (Fig. 8B, Fig. 9A) would affect cyst formation in *double bubble* mutants. Treating *dbb*-mutant embryos with 100 μM DAPT at 9.5 hpf significantly reduced cyst formation (Fig. 10I,J). These results strengthen our previous findings that motile cilia are key organelles in fluid transport in the pronephros (Kramer-Zucker et al., 2005) and further suggest that the capacity of the kidney for fluid transport can be modulated by Notch-mediated control of the number of multiciliated versus transporting cells during a crucial stage of kidney-nephron patterning.

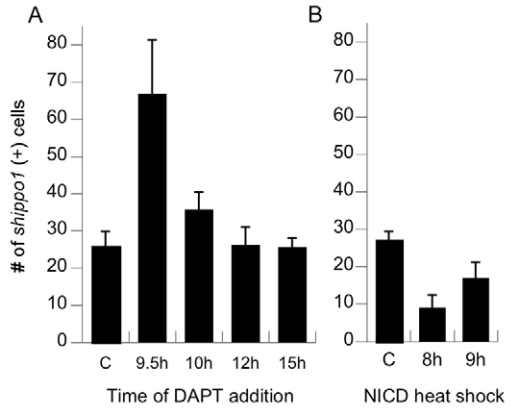

Fig. 9. Notch signaling acts in a short time-window early in pronephric development. *shippo1*-positive multiciliated cells were counted at 34 hpf (in situ hybridization) in individual tubules. (**A**) Quantification of MCCs per pronephric tubule in experiments adding DAPT at progressively later times. (**B**) Quantification of MCCs per pronephric tubule in embryos induced to express Notch1a ICD by heat shock at 8 hpf and 9 hpf. See text for details.

DISCUSSION

Like all epithelial organs, the physiological activity of the zebrafish pronephros is dependent on a partitioning of epithelial tubules into segments and cell types with unique activities that combine to achieve overall organ function. Kidney cells are specialized for osmotic homeostasis and electrolyte balance, which, in a freshwater vertebrate, translates to excretion of water and avid recovery of filtered ions and metabolites (Hickman and Trump, 1969). In the pronephros, multiciliated cells form the biological equivalent of a 'screw pump' by beating in a helical wave pattern in a tight-fitting tubule lumen to propel fluid out of the pronephric opening (Kramer-Zucker et al., 2005), while transporting epithelial cells recover specific ions from filtered blood depending on the spectrum of ion transporters that they express. Very little is known about how kidney-tubule cells achieve their different identities. What we have shown is that a patterning mechanism underlying cell-type specification in the pronephros involves Jagged 2/Notch signaling. Our findings point to a lateral inhibition mechanism in generating a 'salt-and-pepper' pattern of multiciliated cells intercalated among transporting cells.

Notch signaling and kidney development

Previous studies on mouse kidney development have demonstrated roles for Notch signaling in glomerulus formation and in specifying the fates of proximal versus distal nephron tubule segments. Notch2 expression in glomerular epithelial cells and Jagged 1 expression in endothelial cells is required for development of the glomerular vasculature (McCright, 2003). Defects in glomerular vasculature structure have also been reported in combined *jagged 2/jagged 1b* morphants in zebrafish, suggesting that similar developmental mechanisms may underlie zebrafish pronephric development (Lorent et al., 2004). General inhibition of Notch signaling by gamma-secretase inhibition or by mutations in mouse presenilin genes results in a loss of both glomerular and proximal tubule cells, and in the preservation of the distal nephron fate (Cheng et al., 2003). Jagged 1 signaling has also been shown to regulate branching morphogenesis of the renal collecting system (Kuure et al., 2005). In pronephric development in *Xenopus*, Notch signaling also regulates the patterning of proximal versus distal tubule types (McLaughlin et al., 2000). Together, these studies point to a role for Notch signaling in nephron precursor structures (S-shaped bodies or their equivalents) in broadly defining major nephron segments and structures.

Our focus on Jagged 2/Notch3 signaling in the zebrafish introduces an additional role for Notch signaling in the generation of intercalated cell patterns in the nephron. In the mammalian collecting system, 'intercalated cells' exist interspersed among principal cells, and function in pH homeostasis (Seldin and Giebisch, 1992). Although mechanisms regulating intercalated cell differentiation are currently unknown, the expression of multiple notch and jagged genes in the nephron suggests that notch signaling could play a role (Challen et al., 2006). In the zebrafish larval intestine, secretory and adsorptive cells exist intermixed, and this balance of cell types is controlled by a Notch-mediated lateral inhibition mechanism involving *deltaD* expression in secretory cells (Crosnier et al., 2005). In the zebrafish liver, the hepatocyte/cholangiocyte cell-fate choice also appears to be under the control of Notch signaling (Lorent et al., 2004). In the pancreas, disruption of Notch signaling by mutation in *mind bomb* or by expression of a dominant-negative *Suppressor of Hairless* construct accelerates differentiation of exocrine acinar cells (Esni et al., 2004). The picture that emerges from these studies is that Notch signaling may act at multiple stages of organogenesis to first regulate the timing and overall pattern of organ progenitor cell differentiation, and may later serve to modify cell-type specification by superimposing a repression mechanism that regulates the proportion of different cell types in the mature organ.

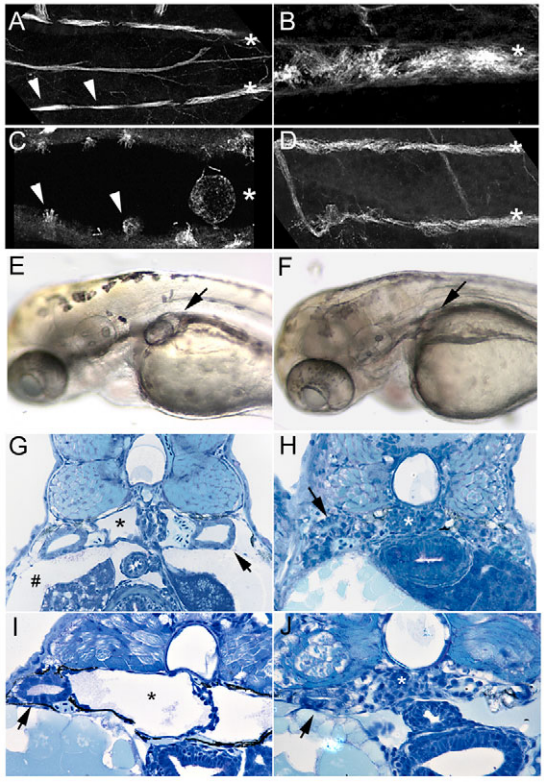

Fig. 10. Enhanced ciliogenesis and *double bubble*-mutant rescue by inhibition of Jagged/Notch signaling. (**A**) Wild-type *double bubble* (*dbb*) heterozygotes viewed in confocal projections show alternating thick acetylated tubulin-positive bundles of cilia (arrowheads) in pronephric lumens (individual tubules are marked with asterisks in A-D). (**B**) *jagged 2* exon 20-morpholino injection results in enhanced ciliogenesis, seen here in a single pronephric tubule with a slightly expanded lumen. (**C**) A single *dbb*-mutant pronephric lumen showing loss or shortening of cilia bundles (arrowheads), and a grossly dilated lumen (*). (**D**) *dbb*-mutant homozygotes injected with *jagged 2* exon 20 morpholino recover normal tubule morphology (paired tubules marked with asterisks) and show a dramatic recovery of ciliogenesis. (**E**) *dbb*-mutant homozygote at 2.5 dpf showing bilateral cyst formation in the proximal pronephros (arrow). (**F**) *dbb*-mutant homozygote injected with *jagged 2* exon 20 morpholino at the one-cell stage, showing the absence of cyst formation (arrow; mutant rescue). (**G**) Histological section of *dbb*-homozygote pronephros showing a cystic pronephric tubule (*), dilated pronephric tubules (arrow) and edema (#). (**H**) Histological section of a *dbb* homozygote injected with *jagged 2* exon 20 morpholino at the one-cell stage, showing complete absence of cystic pathology and edema (arrow, wild-type-appearing pronephric tubules; *, normal glomerular structure). (**I**) Control DMSO-treated *dbb*-mutant embryo showing cystic proximal pronephros (*). (**J**) DAPT treatment (100 μM started at 9.5 hpf) of *dbb* homozygotes eliminates cystic distension of the pronephros (*, glomerulus; arrow, pronephric tubule). In these experiments, *dbb* homozygotes were identified independently of cyst formation by the characteristic mutant ventral axis curvature in 25% of embryos from heterozygote pair matings.

Notch signaling pathways and the emergence of multiciliated cells

The work presented here suggests that, early in pronephric cell differentiation (8-10 hpf), cells expressing high levels of Jagged 2 interact with neighboring Notch-expressing cells (summarized in Fig. 11). Jagged 2/Notch signaling requires the E3 ligase *mind bomb*, and initiates proteolysis of Notch3 and the liberation of the transcriptionally active NICD. The NICD activates expression of unknown repressors that downregulate *rfx2* (and downstream cilia genes) and *jagged 2*. Later in development, a subset of cells becomes multiciliated while neighboring cells acquire a transporting-epithelial-cell fate and express ion channel genes.

In other Notch signaling contexts, the hairy/enhancer of split family of genes exert the transcriptional-repressor function of Notch signaling (Iso et al., 2003). We have found that *her9* is expressed in the pronephros (see Fig. S2 in the supplementary material); however, it is clear that other factors must act to repress ciliogenic gene expression, because the expression of *rfx2* and *shippo1* was unaffected by a *her9*-translation-blocking morpholino (Y.L. and I.A.D., unpublished). Whatever the identity of Notch-induced transcriptional repressors may be, it is likely that the transcription factor *rfx2* may be a primary target of repression, because it belongs to a gene family known to serve as master regulators of ciliogenic

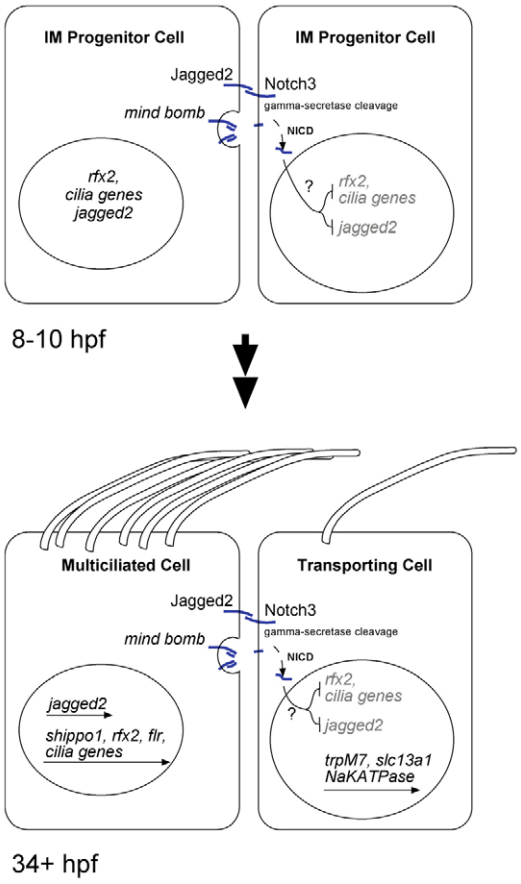

Fig. 11. A model of Jagged/Notch regulation of cell-type specification in the pronephros. Early in pronephric cell differentiation (8-10 hpf), cells expressing high levels of Jagged 2 interact locally with Notch3-expressing cells to inhibit the expression of *rfx2* and *jagged 2*. Jagged 2/Notch3 signaling requires the E3 ligase *mind bomb*, and initiates regulated intramembranous proteolysis (RIP) of Notch3 and the liberation of the transcriptionally active Notch3 intracellular domain (N(3)ICD). N(3)ICD activates expression of unknown repressors (?), which downregulate *rfx2* (and downstream cilia genes) and *jagged 2*. Later in development (assayed here at 34-56 hpf), a subset of cells becomes multiciliated and N(3)ICD-mediated transcriptional regulation in neighboring cells directly or indirectly regulates the expression of ion channel genes and allows these cells to acquire a transporting-epithelial-cell fate.

gene expression in organisms ranging from *C. elegans* to mammals (Bonnafe et al., 2004; Efimenko et al., 2005; Swoboda et al., 2000). Repression of *rfx2* in neighbors of MCC would be expected to restrict abundant ciliogenic gene expression to the MCCs and limit MCC number. This idea is supported by our finding that *rfx2* morpholino injection results in pronephric cyst formation and in an absence of motile pronephric cilia (Y.L. and I.A.D., unpublished).

Our work has also identified a negative-feedback loop in which Jagged 2 signaling from MCCs directs the repression of *jagged 2* transcription in neighboring cells. As originally proposed for neurogenic gene signaling in *Drosophila* (Goriely et al., 1991; Heitzler and Simpson, 1991), this cell interaction would be predicted to exaggerate differences between neighboring cells in the intermediate mesoderm, where *jagged 2* is first expressed in kidney cells, and to drive the initial emergence of MCC progenitors from a common pool of cells.

Although we have characterized several elements of Notch signaling in pronephric patterning, aspects of our data indicate that the framework is incomplete. In embryos in which Notch signaling has been generally inhibited (i.e. *mind bomb* mutants and DAPT-treated embryos), the transfating of cells to MCCs is observed not only in the pronephric distal segment but also in the proximal segment, where MCC cells are not as frequently seen, suggesting that additional notch genes or Notch ligands may regulate MCC differentiation. These factors are unlikely to include *jagged 1b*, because single knockdown of *jagged 1b* or double knockdown of *jagged 1b* and *jagged 2* did not replicate the *mind bomb* phenotype (data not shown).

Development and functions of cilia

The importance of cilia in normal organ function is highlighted by the pathology observed in organisms with non-functional cilia: kidney cystic disease, retinal degeneration, organ laterality defects and hydrocephalus (Pazour, 2004). Mutations in a variety of cilia-associated proteins cause kidney tubules to become cystic (Barr et al., 2001; Blacque et al., 2004; Fan et al., 2004; Haycraft et al., 2001; Kim et al., 2004; Kramer-Zucker et al., 2005; Morgan et al., 2002; Murcia et al., 2000; Mykytyn et al., 2004; Otto et al., 2003; Pazour et al., 2000; Pazour and Rosenbaum, 2002; Qin et al., 2001; Sun et al., 2004; Yoder et al., 2002). In cultured mammalian epithelial cells, cilia are proposed to function as non-motile fluid-flow sensors that regulate epithelial responses to flow via calcium signaling (Nauli et al., 2003; Praetorius and Spring, 2001). We have demonstrated a role for motile cilia in cystic kidney pathology that affects the zebrafish pronephros (Kramer-Zucker et al., 2005). Multiciliated cells similar to what we describe here in the zebrafish have also been shown to exist intercalated between proximal tubule cells in the human kidney (Duffy and Suzuki, 1968; Hassan and Subramanyan, 1995; Katz and Morgan, 1984; Ong and Wagner, 2005). The function of these cells is currently unknown. Although there is no evidence for Notch signaling in the development of these cells, studies of epidermal development in *Xenopus* have highlighted a role for Notch signaling in the context of ciliated-cell development. The regularly spaced pattern of MCC differentiation in the *Xenopus* epidermis has been shown to be regulated by X-Delta-1 expression in a subset of epidermal cells, which later abundantly express alpha-tubulin and become multiciliated (Deblandre et al., 1999). Taken together with our work, the results suggest that Notch signaling might be a general feature of specialized ciliated cell development.

Our finding that *jagged 2* knockdown can rescue cilia assembly in the *double bubble* mutant and prevent cyst formation strengthens our previous results that cilia-driven fluid flow in the pronephros is a key factor in cystic pathology. This result also raises questions concerning the process of ciliogenesis and the nature of the *dbb* mutation. It is not clear at present why only *dbb* and not other ciliogenesis mutants are rescued by *jagged 2* knockdown. The *dbb* allele we are studying (m468) could be a hypomorphic allele or, alternatively, the *dbb* protein may not be absolutely essential for ciliogenesis. Other intraflagellar transport (IFT) proteins, if expressed in high enough amounts, may be sufficient to compensate for the loss of *dbb*. Further characterization of the *dbb* mutation and its interactions with other proteins involved in IFT will be necessary to resolve these issues.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/6/1111/DC1

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