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Wnt/β-catenin signaling directly regulates Foxj1 expression and ciliogenesis in zebrafish Kupffer's vesicle

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SUMMARY

Cilia are essential for normal development. The composition and assembly of cilia has been well characterized, but the signaling and transcriptional pathways that govern ciliogenesis remain poorly studied. Here, we report that Wnt/β-catenin signaling directly regulates ciliogenic transcription factor *foxj1a* expression and ciliogenesis in zebrafish Kupffer's vesicle (KV). We show that Wnt signaling acts temporally and KV cell-autonomously to control left-right (LR) axis determination and ciliogenesis. Specifically, reduction of Wnt signaling leads to a disruption of LR patterning, shorter and fewer cilia, a loss of cilia motility and a downregulation of *foxj1a* expression. However, these phenotypes can be rescued by KV-targeted overexpression of *foxj1a*. In comparison to the FGF pathway that has been previously implicated in the control of ciliogenesis, our epistatic studies suggest a more downstream function of Wnt signaling in the regulation of *foxj1a* expression and ciliogenesis in KV. Importantly, enhancer analysis reveals that KV-specific expression of *foxj1a* requires the presence of putative Lef1/Tcf binding sites, indicating that Wnt signaling activates *foxj1a* transcription directly. We also find that impaired Wnt signaling leads to kidney cysts and otolith disorganization, which can be attributed to a loss of *foxj1* expression and disrupted ciliogenesis in the developing pronephric ducts and otic vesicles. Together, our data reveal a novel role of Wnt/β-catenin signaling upstream of ciliogenesis, which might be a general developmental mechanism beyond KV. Moreover, our results also prompt a hypothesis that certain developmental effects of the Wnt/β-catenin pathway are due to the activation of Foxj1 and cilia formation.

KEY WORDS: Wnt/β-catenin signaling, Ciliogenesis, Foxj1, Kupffer's vesicle Zebrafish

INTRODUCTION

Cilia are microtubule-based organelles that are assembled and maintained by intraflagellar transport (IFT) proteins (Pedersen and Rosenbaum, 2008). Cilia can be broadly divided into sensory and motile subtypes (Satir and Christensen, 2007). Sensory or primary cilia form on nearly all interphase and nondividing cells (Pedersen and Rosenbaum, 2008) and are the site of transduction for developmental signaling pathways such as the Hedgehog (Hh) pathway and possibly the Wnt pathways (Eggenschwiler and Anderson, 2007). By contrast, motile cilia almost exclusively form in tissues that produce fluid flow, such as the respiratory airways, spinal canal and embryonic node (Eley et al., 2005). Genetic defects in motile cilia lead to primary ciliary dyskinesia (PCD), a heterogeneous syndrome that is characterized by respiratory infections, hydrocephalus, situs inversus, male infertility and occasionally cystic kidney and retinal degeneration (Afzelius, 1976; Olbrich et al., 2002; Kramer-Zucker et al., 2005; Badano et al., 2006). Motile cilia biosynthesis specifically requires Foxj1, a winged-helix domain-containing transcriptional factor (Chen et al., 1998). Recently, Foxj1 has been demonstrated to not only be necessary, but also sufficient for motile cilia synthesis (Stubbs et al., 2008; Yu et al., 2008). However, little is known about the molecular mechanism that governs *foxj1* expression.

In a developing zebrafish embryo, motile cilia are present in cells lining the Kupffer's vesicle (KV), a temporary organ equivalent to the mouse node and to *Xenopus* gastrocoel roof plate (GRP). These nodal cilia rotate in a clockwise direction to generate a leftward flow of extracellular fluid that is essential for the establishment of left-right (LR) asymmetry (Nonaka et al., 1998; Nonaka et al., 2002; Essner et al., 2005; Kramer-Zucker et al., 2005; Hirokawa et al., 2006). Uniquely in zebrafish, fate-mapping studies have shown that KV originates from a group of dorsal forerunner cells (DFCs) (Cooper and D'Amico, 1996; Melby et al., 1996). These DFCs retain cytoplasmic bridges with the yolk cell after other cells are no longer connected to the yolk. Consequently, genetic manipulation can be achieved exclusively in DFCs of zebrafish (Amack and Yost, 2004).

Motile cilia have also been found in the developing zebrafish pronephric ducts (PDs), otic vesicles (OVs) and floor plate (FP) (Kramer-Zucker et al., 2005). PD motile cilia are required for normal kidney organogenesis and ciliary motility in OVs ensures proper formation of otoliths, which are important for the ear's sensory function (Riley et al., 1997; Kramer-Zucker et al., 2005; Yu et al., 2008; Colantonio et al., 2009; Yu et al., 2011). Two distinct *foxj1* genes, *foxj1a* and *foxj1b*, have been identified in zebrafish tissues. Their expression is mutually exclusive in KV (*foxj1a*) and OVs (*foxj1b*), but partially overlaps in PDs and FP (Aamar and Dawid, 2008; Yu et al., 2008). Depletion of *foxj1a* results in abnormal LR asymmetry and renal cyst formation, whereas depletion of *foxj1b* leads to otolith disorganization (Stubbs et al., 2008; Yu et al., 2008; Yu et al., 2011). Whether *foxj1* expression and cilia synthesis in different tissues are regulated by a general developmental mechanism remains to be investigated.

The Wnt/β-catenin pathway is activated upon binding of Wnt ligands to the Frizzled–low-density lipoprotein receptor-related protein (Fzd-LRP5/6) receptor complex. This causes β-catenin stabilization and translocation to the nucleus where it binds to the lymphoid enhancer factor and T cell factor (Lef and Tcf) transcription factors to activate Wnt target gene expression (Logan

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and Nusse, 2004; Willert and Jones, 2006). Wnt/β-catenin signaling has been implicated in the establishment of LR asymmetry. Moderate upregulation of Wnt signaling that does not alter dorsoanterior structure leads to no-looping hearts as seen in the zebrafish *apc* and *mbl* mutants (Carl et al., 2007; Lin and Xu, 2009); however, it has no effect on left-sided *spaw* expression, suggesting that LR patterning function of KV is not affected (Carl et al., 2007; Lin and Xu, 2009). Downregulation of Wnt signaling, as seen in mouse *Wnt3a* mutants and zebrafish *wnt3a* and *wnt8a* morphants, causes randomized organ laterality and randomized side-specific gene expression, which is likely to be a combined effect of Wnt activity on midline formation and KV function (Nakaya et al., 2005; Lin and Xu, 2009). The precise function of Wnt signaling in KV has yet to be elucidated.

Here, using zebrafish KV development as a paradigm, we aim to elucidate molecular mechanisms of how Wnt/β-catenin signaling regulates LR asymmetry. Our data reveal a temporal and KV cell-autonomous Wnt function in LR axis determination and cilia formation. Importantly, we show that this line of Wnt function can be ascribed to its direct transcriptional regulation of *foxj1a*. We also expand our studies to the developing PDs and OVs, two epithelia that require Wnt activity for their normal development and function. Our results indicate that reduction of Wnt signaling impairs *foxj1* expression and ciliogenesis in both of these tissues, suggesting a more general role for the Wnt/β-catenin pathway in the control of *foxj1* expression and cilia formation.

MATERIALS AND METHODS

Zebrafish strains

Wild-type (TL), *Tg(hsp:β-catenin-GFP)* and *Tg(hsp:dkk1-GFP)* strains of zebrafish were used for this work. The two heat-inducible transgenic lines were generated by injection of *hsp70* promoter-driven *dkk1* or *β-catenin1* expression plasmids into one-cell staged embryos. Heat shock was carried out at 40°C for 60 minutes, unless otherwise specified in the Results.

To generate the *Tg(0.6foxj1a:gfp)* transgenic reporter line, a 0.6 kb fragment that is located approximately –5.2 kb to –4.6 kb upstream of the ATG start codon of the zebrafish *foxj1a* gene was inserted into a *tol2* GFP vector. The resulting plasmids were co-injected with *tol2* transposase RNA into one-cell staged embryos. To generate the *Tg(0.6Δfoxj1a:gfp)* transgenic reporter strain, all three putative Lef1/Tcf binding sites (CTGTT, CCTTTGTT and CACAG) in the 0.6 kb fragment were deleted using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent).

Morpholino injections

Antisense morpholino oligonucleotides (MOs) against *β-catenin1* (*ctmnb1* – Zebrafish Information Network) (Lyman Gingerich et al., 2005), *fgfr1* (Neugebauer et al., 2009) and *fzd10* (targeting the AUG site: 5′-TGAGTCCGACACCGGCAGCAAACAT-3′) were obtained from Gene Tools, LLC. Unless specified otherwise in the Results, 4 ng of each morpholino was used. To target morpholinos specifically to DFCs, lissamine-tagged MOs were injected into the yolk cell at ~512-cell stage as previously described (Amack and Yost, 2004). The lissamine tag allowed us to then select embryos in which the dye had diffused throughout the yolk cell and into DFCs.

Cloning and RNA injections

Full-length zebrafish *axin1*, *foxj1a* and *fgf8a* cDNA were amplified by an Expand High-Fidelity PCR system (Roche), using zebrafish cDNA as a template. The resulting cDNA fragments were cloned into the *pCS2+* plasmid.

Capped mRNAs were synthesized from the above *pCS2+* plasmids and *pCS2-wnt8a* construct (Lin et al., 2007) using the SP6 mMESSAGE mMACHINE kit (Ambion). To achieve DFC-specific over-expression, 4 ng of *axin1* RNA, 2 ng of *foxj1a* RNA, 0.2 ng of *wnt8a* RNA or 50 pg of *fgf8a* RNA together with dextran-rhodamine lineage tracing dye (Molecular Probes) or lissamine-tagged MO were injected into the yolk cell at ~512-cell stage.

In situ hybridization

Single-color whole-mount in situ hybridization and two-color fluorescent hybridization were conducted as previously described (Lin and Xu, 2009). *spaw* and *pitx2* proportions were analyzed using Fisher's exact test. Results were considered to be significant when $P<0.01$.

SU5402 treatment

SU5402 treatment was carried out as previously described (Neugebauer et al., 2009).

Antibody staining

Cilia were visualized by immunostaining using monoclonal anti-acetylated tubulin antibody (Sigma) as previously described (Lin and Xu, 2009). Cilia length and number were measured using AxioVision software and analyzed using a two-tailed Student's *t*-test. Results were considered to be significant when $P<0.01$.

Western blotting

Embryos at ~24 hours post-fertilization (hpf) were deyolked for embryo lysate extraction as previously described (Link et al., 2006). Lysates were loaded onto 4-20% Tris-HCl mini gradient gels (BioRad) and transferred to PVDF membranes. Anti-Fzd10 antibody (ProteinTech Group) was used at 1:400, and anti-Actin antibody (Santa Cruz Biotechnology) at 1:1000. Blots were developed using ECL plus Western Blotting Detection System (Amersham).

RESULTS

Sufficient Wnt/β-catenin signaling before segmentation is essential for the establishment of LR asymmetry

Previously, we showed that knocking down Wnt3a or Wnt8a in zebrafish results in cardiac asymmetry defects (Lin and Xu, 2009). Because Wnt/β-catenin signaling plays stage-dependent roles during development (Ueno et al., 2007), we decided to determine the time window during which Wnt signaling is needed for LR axis determination. To temporally reduce Wnt/β-catenin signaling, we generated a transgenic *Tg(hsp:dkk1-GFP)* fish line in which the expression of Dkk1 (Dickkopf1), an antagonist of the Wnt/β-catenin pathway (Glinka et al., 1998), was driven by a heat-shock promoter (supplementary material Fig. S1A,B). We found that Dkk1 activation in embryos from 30% epiboly to bud stage all randomized cardiac jogging. By contrast, induction of Dkk1 from the 3-somite stage onwards did not elicit significant jogging defects (A.C., unpublished). At the molecular level, induction of Dkk1 prior to the 3-somite stage altered left-sided gene expression, including *spaw* in the lateral plate mesoderm (LPM) and *pitx2* in the posterior LPM (Table 1; supplementary material Fig. S1C). As expected, non-transgenic siblings heat-shocked at all stages displayed normal expression of *spaw* and *pitx2* (Table 1). To activate Wnt/β-catenin signaling temporally, we generated a transgenic *Tg(hsp:β-catenin-GFP)* fish line (supplementary material Fig. S1D-F). Induction of β-catenin1 at stages up to 10-somites did not alter either cardiac jogging or *spaw* expression (Table 1) (A.C., unpublished). Thus, we conclude that Wnt/β-catenin signaling must be kept above a certain level before segmentation to ensure the establishment of LR asymmetry.

Wnt/β-catenin signaling regulates LR patterning KV cell-autonomously

To determine whether Wnt signaling affects LR axis determination directly or indirectly via its role in maintaining midline integrity (Takada et al., 1994; Nakaya et al., 2005; Thorpe et al., 2005; Lin and Xu, 2009), we performed DFC-targeted knockdown of genes in the Wnt/β-catenin pathway. We first tested *wnt3a* and *wnt8a*,

Table 1. Stage-dependent effects of Wnt signaling on left-sided gene expression

		spaw					*pitx2*			
Heat-shock stage	*n*	L (%)	R (%)	A (%)	B (%)	*n*	L (%)	R (%)	A (%)	B (%)
Tg(hsp:dkk1-GFP)										
30% epiboly*	120	29	8	4	59	111	34	7	14	44
75% epiboly*	250	38	10	8	44	349	50	17	17	16
bud*	202	53	18	7	22	277	50	19	13	18
3s	94	74	7	6	13	190	85	3	5	7
10s	119	95	–	5	–	72	91	2	5	2
Tg(hsp:β-catenin-GFP)										
30% epiboly	24	94	6	–	–	–	–	–	–	–
bud	28	93	–	–	7	–	–	–	–	–
3s	24	99	1	–	–	–	–	–	–	–
10s	21	98	1	–	1	–	–	–	–	–
Non-transgenic siblings (GFP–)										
30% epiboly	101	98	–	1	1	84	90	1	6	3
75% epiboly	213	87	5	2	6	86	94	4	1	1
bud	115	86	4	3	7	62	94	–	6	–
3s	94	84	7	2	7	127	90	4	3	3
10s	62	91	2	6	1	48	92	–	6	2

Tg(hsp:dkk1-GFP) and *Tg(hsp:β-catenin-GFP)* out-cross embryos were heat activated at the indicated developmental stages, fixed at 21-22 somites, stained and quantified for the percentages of left-sided (L), right-sided (R), absent (A) and bilateral (B) expression of *spaw* or *pitx2*.
n, number of embryos scored.
*$P<0.01$ compared with non-transgenic siblings.

which were previously shown to be expressed near or within KV (Lin and Xu, 2009). Two-color fluorescent in situ hybridization revealed that they had overlapping expression with *charon* (*dand5* – Zebrafish Information Network), a novel member of the Cer/Dan family of Nodal antagonists that has KV-specific expression (Hashimoto et al., 2004), confirming their KV localization (supplementary material Fig. S2I-N). We found that DFC-targeted injection of *wnt3a* or *wnt8a* MOs resulted in LR asymmetry defects (A.C., unpublished). However, we cannot ascribe a KV-cell autonomous function to them because Wnts are secreted molecules. We then turned our attention to frizzled receptors. We screened 12 annotated zebrafish frizzled homologs and found the expression of *fzd10*, which encodes a putative receptor for Wnt3a and Wnt8a (Momoi et al., 2003; Kemp et al., 2007), near or within KV (Fig. 1B,C). *fzd10* expression can be detected in DFCs at ~80% epiboly (Fig. 1A, arrow), and in the brain, dorsal neural tube and tail bud/KV at the 10-somite stage (Fig. 1B,C) (see also Nasevicius et al., 2000). To deplete Fzd10, we designed a translation-blocking MO of *fzd10*. Injection of this MO into one-cell staged embryos greatly suppressed Fzd10 levels, as revealed by western blotting (Fig. 1D). Consistent with the notion that *fzd10* transduces the Wnt/β-catenin pathway (Terasaki et al., 2002; Momoi et al., 2003; Wang et al., 2005), the expression of specific targets of this pathway were inhibited, as demonstrated by depletion of *sp5l* from DFCs in DFC$^{fzd10\ MO}$ embryos (Fig. 1F, arrow) and downregulation of *axin2* in the brain, neural tube and tail bud region in *fzd10* morphants (Fig. 1H) (Jho et al., 2002; Huang and Schier, 2009). Conversely, a role of *fzd10* in the Wnt/PCP pathway, as suggested by studies in synovial sarcomas and synchronous colorectal tumors (Fukukawa et al., 2009; Nagayama et al., 2009), was excluded because defects in convergent extension movement, such as widened somite and abnormal *ntl*, *hgg1* (*cts1b* – Zebrafish Information Network) and/or *dlx3* staining, were not detected in *fzd10* morphants (supplementary material Fig. S2O-R). As expected, asymmetry defects were observed in *fzd10* morphants (Fig. 1J) and, more importantly, in DFC$^{fzd10\ MO}$ embryos. For example, injection of 1 ng of MO resulted in a significant defect in *spaw* expression (50% left, 20% right, 1% absence, 29% bilateral; n=119) compared with those containing *fzd10* MO in the yolk alone (95% left, 2% right, 3% bilateral; n=78) (Fig. 1I,J; $P<1\times10^{-10}$). Injection of 4 ng of MO caused more severe defects (35% left, 35% right, 30% bilateral; n=63). These data suggest a KV-cell autonomous Wnt function in LR axis determination.

To consolidate this notion, we expanded our search to other components of the Wnt/β-catenin pathway and detected near or within KV expression of intracellular Wnt signal transducers *axin2*, *β-catenin1* and *β-catenin2* (*ctnnb2* – Zebrafish Information Network) as well as transcription factors *lef1* and *tcf7* (supplementary material Fig. S2D-H). DFC$^{axin1\ RNA}$ embryos showed moderate, but statistically significant, alterations in *spaw* expression (Fig. 1J; $P<2\times10^{-04}$ compared with Yolk$^{axin1\ RNA}$ embryos). Moreover, DFC$^{\beta\text{-}catenin1\ MO}$ embryos also exhibited significant randomization in *spaw* expression relative to the Yolk$^{\beta\text{-}catenin1\ MO}$ embryos (Fig. 1J; $P<4.37\times10^{-05}$). Collectively, our data indicate that Wnt signaling has an intrinsic function in LR axis determination.

Wnt/β-catenin signaling regulates ciliogenesis in KV

To gain insight into how Wnt signaling regulates LR patterning, we examined ciliogenesis in KV. We found that induction of Dkk1 at stages prior to the 3-somite stage led to a significant reduction in cilia length. Specifically, moderate Dkk1 induction (Dkk+) by heating *Tg(hsp:dkk1-GFP)* embryos for 30 minutes resulted in shorter cilia (4.38±0.56 μm) compared with heat-shocked non-transgenic siblings (5.46±0.47 μm) (Fig. 2A,B,J; $P<3\times10^{-04}$) and wild-type embryos (5.67±0.37 μm) (Fig. 2D,J; $P<1.34\times10^{-06}$), both of which had similar cilia length ($P<0.43$). The number of cilia in Dkk+ embryos remained normal (36±13 relative to 42±12 in non-transgenic siblings) (Fig. 2A,B,K; $P<0.32$). However, if a

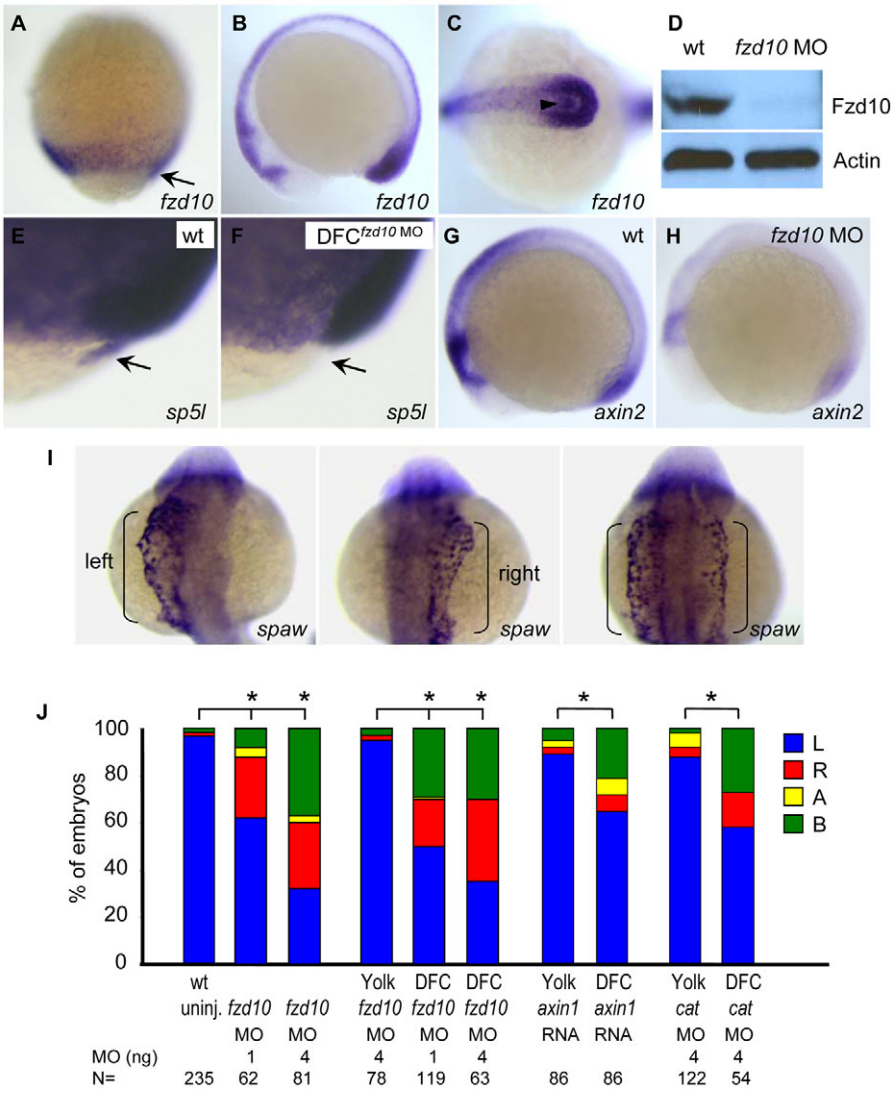

Fig. 1. Wnt/β-catenin signaling regulates LR asymmetry KV cell-autonomously. (**A-C**) *fzd10* expression pattern. Shown are a lateral view of 80% epiboly (A), and lateral (B) and dorsal (C) views of the tail bud region of 10-somite staged zebrafish embryos with anterior to the left. Arrow, DFCs; arrowhead, KV. (**D**) Western blot showing diminished Fzd10 level in *fzd10* morphants. Actin was used as a loading control. (**E-H**) *fzd10* transduces Wnt/β-catenin signaling. DFC-specific injection of *fzd10* MO depleted *sp5l* expression from DFCs (F). Knockdown of Fzd10 resulted in downregulation of *axin2* expression (H). Shown are lateral views of 80% epiboly (E,F) and 10-somite (G,H) staged embryos. Arrow indicates DFCs. (**I**) Representative images of *spaw* expression (bracket) in DFC$^{fzd10\ MO}$ embryos at the 21-somite stage. (**J**) DFC/KV-specific reduction of Wnt/β-catenin signaling randomizes *spaw* expression. Percentages of *spaw* expression were determined in 21-somite staged embryos. MO (ng), amount of MO used; N, number of embryos examined. L, left-side; R, right-side; A, absence; B, bilateral. *P<0.01 compared with corresponding modulations in the yolk only or with uninjected controls.

higher level of Dkk1 (Dkk++) was induced by increasing the duration of heat shock to 60 minutes, not only was cilia length further shortened (3.04±0.34 μm; $P<8.61\times10^{-15}$), but the number of cilia was significantly reduced as well (23±11; $P<2.0\times10^{-4}$) compared with non-transgenic siblings (Fig. 2C,J,K). Similarly, injection of *fzd10* MO into one-cell staged embryos inhibited cilia length/number in a dose-dependent manner (Fig. 2J,K). By contrast, transient upregulation of Wnt signaling by inducible β-catenin1 expression had no significant effect on cilia length (Fig. 2J; $P<0.58$) or cilia number (Fig. 2K; $P<0.14$) compared with controls. Thus, we conclude that Wnt signaling is required for ciliogenesis in KV, which temporally correlates with its stage-dependent function in regulating LR patterning.

DFC-targeted reduction of Wnt signaling also disrupted ciliogenesis. Cilia in DFC$^{axin1\ RNA}$ embryos were shorter (Fig. 2J; $P<8.95\times10^{-10}$), but the number remained unchanged (Fig. 2K; $P<0.69$) compared with controls. DFC$^{\beta\text{-}catenin1\ MO}$ embryos had shorter ($P<7.99\times10^{-14}$) and fewer ($P<4.88\times10^{-05}$) cilia (Fig. 2J.K). Furthermore, injection of 1 ng of *fzd10* MO significantly reduced cilia length only ($P<5.00\times10^{-11}$), whereas injection of 4 ng of MO reduced both cilia length ($P<1.94\times10^{-14}$) and cilia number ($P<14.07\times10^{-05}$) compared with controls (Fig. 2E,F,J,K). These data indicate a KV cell-autonomous control of cilia formation by Wnt signaling. To explore whether cilia motility is affected, we assessed the expression of *dnah9* (*dynein axonemal heavy polypeptide 9*, also known as *lrdr1*). *dnah9* is the zebrafish homolog of the mammalian *Iv* (*inversus viscerum*; now known as *Dnahc11*) gene that encodes a conserved dynein heavy chain required for cilia motility (Supp et al., 1997; Essner et al., 2005). Dkk1 induction and DFC-targeted injection of MOs against *β-catenin1* or *fzd10* all suppressed *dnah9* expression (Fig. 2H,I and Fig. 3F), suggesting an impairment in cilia motility.

To address whether the abnormal ciliogenesis is secondary to general effects of Wnt signaling on patterning, we assessed KV morphogenesis. First, the expression of *sox17*, a marker of DFCs/KV lineage (Alexander et al., 1999), was evaluated at the bud stage for DFC specification and at 6-somite stage for KV cell maintenance. Neither Dkk1 activation nor DFC-specific injection of *fzd10* MO markedly altered *sox17* expression (supplementary material Fig. S3A-F). Second, we noted that embryos overexpressing Dkk1 and embryos depleted of Fzd10 had mostly normal KV morphology, despite being smaller when a higher level of Dkk1 was induced or a higher dose of MO was used (supplementary material Fig. S3G-I) (A.C., unpublished). Third, we found that the tight junction marker ZO1 was expressed normally in Dkk1-expressing embryos and DFC$^{fzd10\ MO}$ embryos

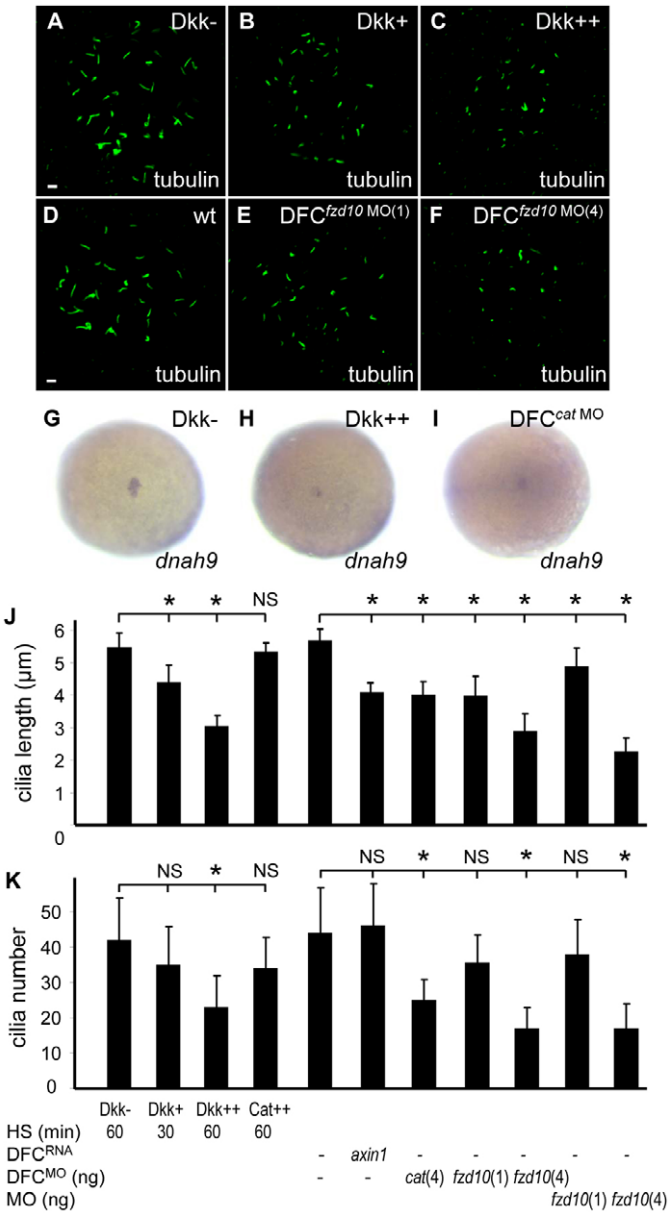

Fig. 2. Reduction of Wnt signaling results in shorter and fewer cilia in KV. (**A-F**) Dose-dependent effect of Wnt signaling on cilia length and number. (A-C) *Tg(hsp:dkk1-GFP)* fish were bred with wild-type fish. Their progenies were heat shocked at 60% epiboly for 30 minutes (B) or 60 minutes (C) to induce Dkk1 expression. Moderate induction of Dkk1 (Dkk+) resulted in shorter cilia (B) whereas higher level of Dkk1 induction (Dkk++) led to shorter and fewer cilia (C). (D-F) DFC-targeted injection of 1 ng of *fzd10* MO reduced cilia length, but not number (E); 4 ng of MO reduced both cilia length and number (F). Cilia were visualized by anti-acetylated tubulin antibody staining of 10-somite staged embryos. Scale bars: 5 μm. (**G-I**) Wnt signaling is essential for *dnah9* expression in DFCs. Dkk1 induction (H) or DFC-targeted injection of *β-catenin1* MO (I) downregulated *dnah9* expression. Shown are ventral views of 95% epiboly staged embryos. (**J**,**K**) Quantification of cilia length (J) and number (K) in 10-somite staged embryos. Approximately 12-16 embryos were analyzed for each group. *Tg(hsp:β-catenin-GFP)* fish were bred with wild-type fish. Their progenies were heat shocked at 60% epiboly for 60 minutes to induce β-catenin1 (Cat++) expression. Data are represented as mean±s.d. **P*<0.01. NS, not statistically significant.

(supplementary material Fig. S3I). Together, these results indicate that Wnt signaling regulates ciliogenesis without disturbing DFC specification, KV formation or apical-basal polarization of KV cells. However, we cannot rule out the possibility that other aspects of KV development might be affected.

The function of Wnt/β-catenin signaling in KV ciliogenesis is mediated by *foxj1a*

Because Wnt/β-catenin signaling functions mainly through transcriptional regulation of its targets, we reasoned that it might modulate genes implicated in KV ciliogenesis. The expression of ciliogenic transcription factor *foxj1a* is initiated in DFCs, becomes robust towards the end of gastrulation (Aamar and Dawid, 2008; Yu et al., 2008; Tian et al., 2009) and is then gradually downregulated in KV (A.C., unpublished). The correlation between *foxj1a* expression in DFCs/KV lineage and the temporal requirement for Wnt signaling in KV ciliogenesis prompted us to examine *foxj1a* transcripts. *foxj1a* expression was reduced in embryos expressing a moderate level of Dkk1 (30 minutes heat shock) and embryos injected with 1 ng of *fzd10* MO (A.C., unpublished), and was nearly abolished in embryos expressing a higher level of Dkk1 (60 minutes heat shock) (Fig. 3B) and embryos injected with 4 ng of *fzd10* MO (Fig. 3C). By contrast, the level of *foxj1a* remained normal following brief upregulation of Wnt signaling via β-catenin1 activation (Fig. 3D).

To determine whether Wnt signaling controls ciliogenesis and LR asymmetry through *foxj1a*, we performed *foxj1a* RNA rescue experiments. Unlike whole-body over-expression (Tian et al., 2009), DFC-targeted over-expression of *foxj1a* RNA did not appear to significantly alter left-sided *spaw* expression (Fig. 3K; $P<0.25$ compared with controls). It also did not cause any apparent changes in cilia length ($P<0.65$) and number ($P<0.73$) relative to controls (Fig. 3I,J). However, it significantly restored cilia length in DFC$^{fzd10\ MO}$ embryos (from 3.20±0.59 μm to 4.68±0.31 μm; $P<8.16\times10^{-09}$), DFC$^{\beta\text{-}catenin1\ MO}$ embryos ($P<3.00\times10^{-04}$) and embryos overexpressing Dkk1 ($P<1.83\times10^{-10}$) (Fig. 3I), which is in agreement with the documented role of *foxj1a* in cilia length elongation (Cruz et al., 2010; Yu et al., 2011). To a lesser extent, ectopic expression of *foxj1a* RNA enhanced cilia number in DFC$^{fzd10\ MO}$ embryos (from 18±7 to 25±8; $P<0.02$), DFC$^{\beta\text{-}catenin1\ MO}$ embryos ($P<0.04$) and embryos with activated Dkk1 ($P<0.15$) (Fig. 3J). In addition to cilia length and number, ectopic expression of *foxj1a* RNA was able to restore *dnah9* expression in DFC$^{fzd10\ MO}$ embryos (Fig. 3E-G). Lastly, ectopic expression of *foxj1a* RNA partially rescued abnormal *spaw* expression in DFC$^{\beta\text{-}catenin1\ MO}$ embryos ($P<4.00\times10^{-03}$) and DFC$^{fzd10\ MO}$ embryos ($P<3.00\times10^{-06}$) (Fig. 3K). Together, our results demonstrate a role of *foxj1a* in Wnt-regulated ciliogenesis and LR asymmetry.

Wnt/β-catenin signaling functions downstream of fibroblast growth factor (FGF) signaling in the control of ciliogenesis

Because FGF signaling has also been implicated in the regulation of ciliary growth (Neugebauer et al., 2009), we attempted to understand how these developmentally important pathways converge on ciliogenesis. Consistent with a previous report (Neugebauer et al., 2009), downregulation of FGF signaling by incubation with 10 μm of SU5402, an FGF pathway inhibitor, or injection with 1 ng of *fgfr1* MO resulted in reduced *foxj1a* expression and reduced cilia length, but the number of cilia was maintained (supplementary material Fig. S4). We also found that treatment with 20 μm of SU5402 or injection with 4 ng of *fgfr1* MO led to a greater inhibition of *foxj1a* expression

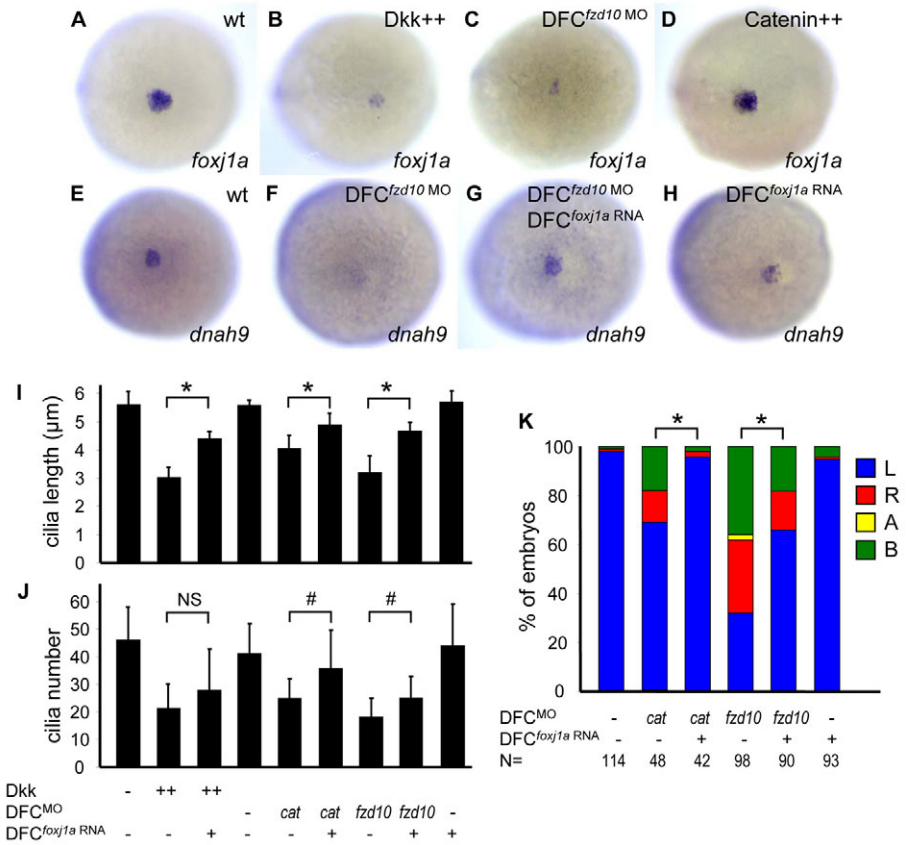

Fig. 3. Wnt signaling regulates KV ciliogenesis through modulation of *foxj1a* expression. (**A-D**) Wnt signaling is required for *foxj1a* expression in DFCs. *foxj1a* expression in DFCs was severely downregulated in Dkk1-expressing zebrafish embryos (B; 37/38) and embryos injected with *fzd10* MO into DFCs (C; 24/27) compared with wild-type controls (A). Induction of β-catenin1 did not appear to affect *foxj1a* expression (D). (**E-H**) Ectopic expression of *foxj1a* restores *dnah9* levels in embryos with impaired Wnt activity. Downregulated *dnah9* expression in DFC$^{fzd10\ MO}$ embryos (F; 16/18) was enhanced by DFC-targeted *foxj1a* overexpression (G; 15/19). Overexpression of *foxj1a* RNA alone did not significantly alter *dnah9* level (H). Shown are ventral views of 90-95% epiboly staged embryos. (**I**,**J**) Ectopic expression of *foxj1a* partially rescues KV cilia length (I) and, to a lesser extent, cilia number (J). Approximately 10-18 embryos for each group were analyzed at the 10-somite stage. Data are represented as mean±s.d. *P<0.01; $^{\#}P$<0.05. NS, not significant (P>0.05). *Tg(hsp:dkk1-GFP)* and *Tg(hsp:β-catenin-GFP)* embryos were heat shocked at 50% epiboly for 60 minutes. (**K**) Ectopic expression of *foxj1a* partially restores left-sided *spaw* expression in embryos with impaired Wnt activity. Percentages of *spaw* expression were determined in 18- to 21-somite staged embryos. N, number of embryos examined. L, left-side; R, right-side; A, absence; B, bilateral.

and reduction of cilia length as well as a reduction in cilia number without significantly affecting DFC specification (Fig. 4B,I,J; supplementary material Fig. S4). These phenotypes are strikingly similar to those induced by blocking of Wnt signaling. Next, the genetic relationship between the Wnt and FGF pathways was examined by epistatic analysis. Although DFC-targeted overexpression of *wnt8a* RNA alone did not have an apparent effect on wild-type embryos (Fig. 4D,I,J), it restored *foxj1a* expression (Fig. 4B,C) and significantly enhanced cilia length (from 2.95±0.58 μm to 4.18±0.46 μm; $P<3.13\times10^{-08}$) (Fig. 4I), and cilia number remained unchanged (Fig. 4J; $P<0.35$) in DFC$^{fgfr1\ MO}$ embryos. By contrast, DFC-targeted overexpression of *fgf8a* RNA failed to rescue *foxj1a* expression (Fig. 4F,G), cilia length (Fig. 4I; $P<0.21$) or cilia number (Fig. 4J; $P<0.24$) in DFC$^{fzd10\ MO}$ embryos. The amount of *fgf8a* RNA used in our rescue experiment was the highest amount that alone did not significantly disrupt either *foxj1a* expression (Fig. 4H) or embryo morphology (A.C., unpublished). Together, our data suggest that Wnt signaling functions downstream of FGF signaling in the regulation of *foxj1a* expression and ciliogenesis in the zebrafish KV.

Wnt/β-catenin signaling directly regulates *foxj1a* transcription in DFCs and KV

We then investigated how Wnt signaling regulates *foxj1a* expression. We first examined the timing between Wnt inhibition/activation and *foxj1a* expression. *Tg(hsp:dkk1-GFP)* embryos were heat shocked at 30% epiboly, the earliest stage showing detectable *foxj1a* expression (Aamar and Dawid, 2008), for 30 minutes and were then collected 30 minutes later for assessment of *foxj1a* expression. Dkk1 induction prevented initiation of *foxj1a* transcription (supplementary material Fig. S5). Conversely, though β-catenin1 activation did not significantly alter *foxj1a* expression 4 hours after heat shock (Fig. 3D), it strongly and transiently enhanced *foxj1a* expression in DFCs 1 hour after heat shock (supplementary material Fig. S5). These rapid responses indicate that Wnt signaling does not require the synthesis of an intermediate protein in order to control *foxj1a* transcription.

To determine whether Wnt signaling directly regulates *foxj1a* transcription, we performed enhancer dissection. Bioinformatics analysis identified a 150 bp stretch of sequence that is homologous with tetradon sequence, 50 bp of which is also conserved in

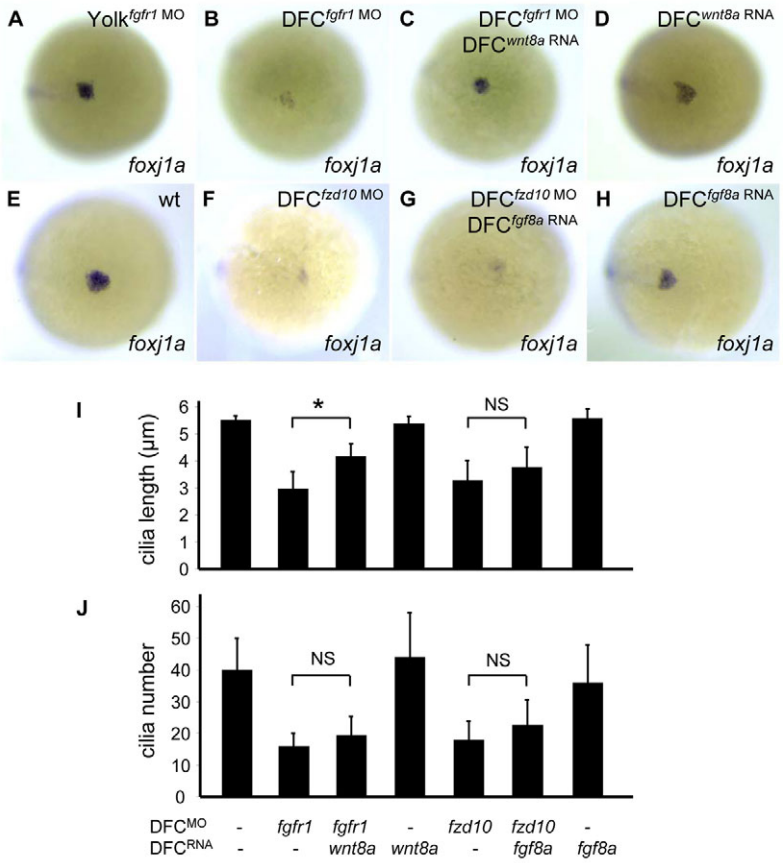

Fig. 4. Wnt signaling functions downstream of the FGF pathway in regulation of *foxj1a* expression and cilia formation. (**A-D**) Ectopic expression of Wnt ligands restores *foxj1a* levels in embryos with reduced FGF signaling. DFC$^{fgfr1\ MO}$ embryos exhibited downregulated *foxj1a* expression in DFCs (B; 17/18) compared with controls (A). Co-injection of *wnt8a* RNA with *fgfr1* MO restored *foxj1a* expression (C; 16/19). Ectopic expression of *wnt8a* RNA alone had no apparent effect on *foxj1a* expression (D). (**E-H**) Ectopic expression of FGF ligands fails to restore *foxj1a* expression in embryos with reduced Wnt signaling. Downregulated *foxj1a* expression in DFC$^{fzd10\ MO}$ embryos (F) was not restored by co-injection of *fgf8a* RNA (G). Ectopic expression of *fgf8a* RNA alone had no apparent effect on *foxj1a* expression (H). Shown are ventral views of 90-95% epiboly staged embryos. (**I**,**J**) Ectopic expression of Wnt ligands partially rescues cilia length defects in embryos with reduced FGF signaling. Shown are quantification of cilia length (I) and cilia number (J). Approximately 12-20 embryos were analyzed for each group. Data are represented as mean±s.d. **P*<0.01. NS, not significant (*P*>0.05).

humans (Fig. 5A). Because the sequence partially overlaps with the predicted first exon, where essential cis-acting elements are typically located in proximity, we generated a series of constructs containing the 150 bp sequence and/or its flanking sequences (Fig. 5A). Two fragments (0.6 kb and 1.6 kb, respectively) were identified to sufficiently direct the expression of GFP fluorescent reporter in KV, PDs and FP by transient injection assay (Fig. 5A) (A.C., unpublished). The smaller 0.6 kb sequence was chosen to generate a transgenic reporter strain. The stable transgenic *Tg(0.6foxj1a:gfp)* embryos expressed *gfp* transcripts in DFCs and GFP in KV, PDs and FP (Fig. 5B,D-F), thus recapitulating endogenous *foxj1a* expression pattern. Importantly, DFC-targeted injection of *fzd10* MO into *Tg(0.6foxj1a:gfp)* embryos abolished *gfp* expression in DFCs (Fig. 5C), suggesting the existence of Wnt-responsive elements in the enhancer region. Upon inspection of the sequence, three putative Lef1/Tcf binding sites were identified (Fig. 5A, red line). We then constructed a GFP reporter plasmid driven by the enhancer with deletions in all three putative Lef1/Tcf binding sites (0.6Δ). In stable transgenic *Tg(0.6Δfoxj1a:gfp)* embryos, GFP reporter expression appeared to be absent from KV and PDs, but was maintained in the FP (Fig. 5G-I), indicating that the putative Lef1/Tcf binding sites are required for tissue-specific *foxj1a* expression. Taken together, we conclude that Wnt signaling directly controls *foxj1a* transcription in KV.

Wnt-controlled ciliogenesis is a general developmental mechanism

To address whether Wnt-Foxj1 signaling regulates ciliogenesis beyond KV, we examined cilia in the developing kidney and inner ear. In the kidney, the Wnt/β-catenin pathway has been implicated in normal development in mice, *Xenopus* and zebrafish (Park et al., 2007; Lyons et al., 2009), and impaired Wnt/β-catenin signaling has been shown to cause cystic kidney in mice (Marose et al., 2008; Lancaster et al., 2009). We found that induction of Dkk1 did not grossly alter the expression of PD precursor marker *pax2* (supplementary material Fig. S6); however, it diminished *foxj1a* expression (Fig. 6B, arrow). Given that *Tg(0.6Δfoxj1a:gfp)* embryos did not exhibit GFP reporter expression (Fig. 5H,I), Wnt signaling might directly regulate *foxj1a* transcription in PDs. Furthermore, induction of Dkk1 resulted in fewer and shorter cilia (4.17±0.49 μm relative to 6.79±0.18 μm in controls; $P<1.03\times10^{-09}$) (Fig. 6G,H,K), as well as a loss of *dnah9* expression in PDs (Fig. 6F, white arrow). Consistent with the cilia defects, cystic distension of PDs was present in day 1 embryos (Fig. 6M, arrow). However, Dkk1 activation did not affect *foxj1a* expression in the FP (Fig. 6B, asterisk), which explains the presence of GFP fluorescent protein in the FP of *Tg(0.6Δfoxj1a:gfp)* embryos (Fig. 5H,I) and suggests a tissue-specific function of Wnt.

The zebrafish ear has two types of cilia: short cilia and tethering cilia. Despite the disagreement regarding which one is the motile type, ciliary motility ensures normal ear development (Riley et al., 1997; Colantonio et al., 2009; Yu et al., 2011). Consistent with our findings in KV and PDs, Dkk1 activation did not alter the expression of otic placode precursor *pax2* (supplementary material Fig. S6), but abolished the expression of *foxj1b* (Fig. 6D, arrowhead) and *dnah9* (Fig. 6F, white arrowhead) in the otic placode. Moreover, induction of Dkk1 resulted in shorter (2.35±0.36 μm relative to 4.09±0.19 μm in controls; $P<1.74\times10^{-08}$) and fewer tethering cilia as well as fewer short cilia (Fig. 6I,J,L). In concordance with the cilia defects, otolith assembly was perturbed. In contrast to the two otoliths positioned at the

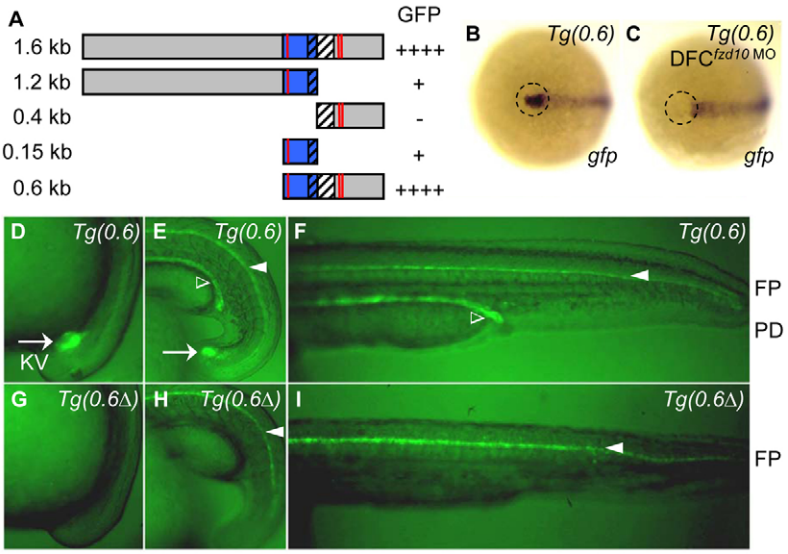

Fig. 5. Wnt signaling directly regulates *foxj1a* transcription. (**A**) Schematic of *foxj1a* enhancers. Sequence of *foxj1a* that is located approximately –6.2 kb to –4.6 kb upstream of the ATG start codon was used to generate a series of report constructs. Blue bar, conserved sequence between zebrafish and tetradon. Hatched bar, predicted first non-coding exon. Red line, putative Lef1/Tcf binding site. These fragments were inserted in front of the *gfp* sequence in a *tol2* vector, and the resulting plasmids were co-injected with *tol2* transposase RNA into embryos. Fluorescent GFP signals in KV were scored. (**B**,**C**) A 0.6 kb enhancer sequence contains Wnt-responsive cis-acting elements. Transgenic *Tg(0.6foxj1a:gfp)* embryos showed reporter *gfp* expression in DFCs (B; 12/12). DFC-targeted injection of *fzd10* MO abolished *gfp* expression in DFCs (C; 14/15). Shown is in situ hybridization using *gfp* as a probe in a ventral view of 95% epiboly staged embryos. Dashed circle indicates DFC region. (**D-I**) Putative Lef1/Tcf binding sites are required for *foxj1a* expression in KV. GFP reporter expression in stable transgenic *Tg(0.6foxj1a:gfp)* embryos recapitulated *foxj1a* expression pattern in KV (D,E), PDs (E,F) and FP (E,F). After all three putative Lef1/Tcf binding sites in the 0.6 kb enhancer were deleted using a site-directed mutagenesis kit, stable transgenic *Tg(0.6Δfoxj1a:gfp)* embryos lacked GFP expression in KV (G,H) and PDs (H,I) but maintained GFP expression in FP (H,I). Arrow indicates KV, open arrowhead PDs and filled arrowhead FP. Shown are embryos at 10-12 somites (D,G), 16 somites (E,H) and 30 hpf (F,I).

anterior and posterior ends of the otic vesicle by 2 days post-fertilization (dpf) in normal embryos, only one otolith was formed (Fig. 6O, arrowhead). These results define an essential role of Wnt/β-catenin signaling in PD and OV ciliogenesis, which is possibly mediated by *foxj1a* and *foxj1b*, respectively. Based on these data, we propose that Wnt-regulated ciliogenesis is a general mechanism during development.

DISCUSSION

Wnt/β-catenin signaling regulates ciliogenesis via *foxj1a*

In this paper, we put forward the novel concept that Wnt/β-catenin signaling regulates ciliogenesis via direct regulation of *foxj1a* transcription. First, we showed that DFC specification and KV morphogenesis are not markedly affected by impaired Wnt signaling, thus arguing that the effect of Wnt signaling on *foxj1a* expression/cilia formation is not secondary to its other functions, such as posterior fate specification (Schier and Talbot, 2005). Second, we placed Wnt signaling genetically downstream of FGF signaling in the regulation of *foxj1a* expression. In the future, similar genetic studies can be extended to examine relationships between the Wnt and other signaling pathways that are also implicated in *foxj1a* expression and/or ciliogenesis, for example, the Notch pathway (Lopes et al., 2010). Third, our finding that the putative Lef1/Tcf binding sites in the *foxj1a* enhancer are indispensable for *foxj1a* expression in KV firmly defines a direct transcriptional regulation of *foxj1a* by Wnt. Our ongoing hypothesis predicts that multiple signaling pathways could regulate ciliogenesis, among which Wnt is responsible for downstream signaling, acting directly at the level of *foxj1a* transcription. Lastly, although Wnt signaling is essential for ciliogenesis, it is not sufficient to induce ectopic *foxj1a* expression. Together with the notion that Wnt signaling has a broader function domain than Foxj1a, we also predict that Wnt signaling needs other co-factors to initiate ciliogenesis. Further analysis of the *foxj1a* enhancer, including searching for crucial cis-acting elements and identification of transcription factors that bind to these elements, will elucidate the transcription circuit that confers Wnt-regulated *foxj1a* expression.

Our data from several motile cilia-forming tissues suggest a general function of Wnt-Foxj1 signaling in governing cilia formation. It remains to be determined whether this function of Wnt is conserved in mammals. We found that two of the three putative Lef1/Tcf binding sites in the zebrafish *foxj1a* enhancer are conserved in the human *FOXJ1* enhancer (Rawlins et al., 2007; Wang and Ware, 2009), suggesting a conserved function. *Wnt3a* knockout mice showed normal ciliogenesis in the node (Nakaya et al., 2005), but this does not necessarily indicate a lack of conservation of the Wnt-Foxj1-ciliogenesis cascade in mice. The following issues need to be addressed before any conclusions can be made. Do other components of Wnt signaling have redundant functions in the node? Is Foxj1 downregulated in the node of Wnt3a-deficient mice or other mouse mutants harboring impaired Wnt signaling? Are there other transcription factors that can compensate for the loss of Foxj1? For example, normal ciliogenesis in the FP of Foxj1-null mice was attributed to redundant functions of Rfx3 (regulatory factor binding to the X box), a protein important for cilia biosynthesis and cilia motility (Bonnafe et al.,

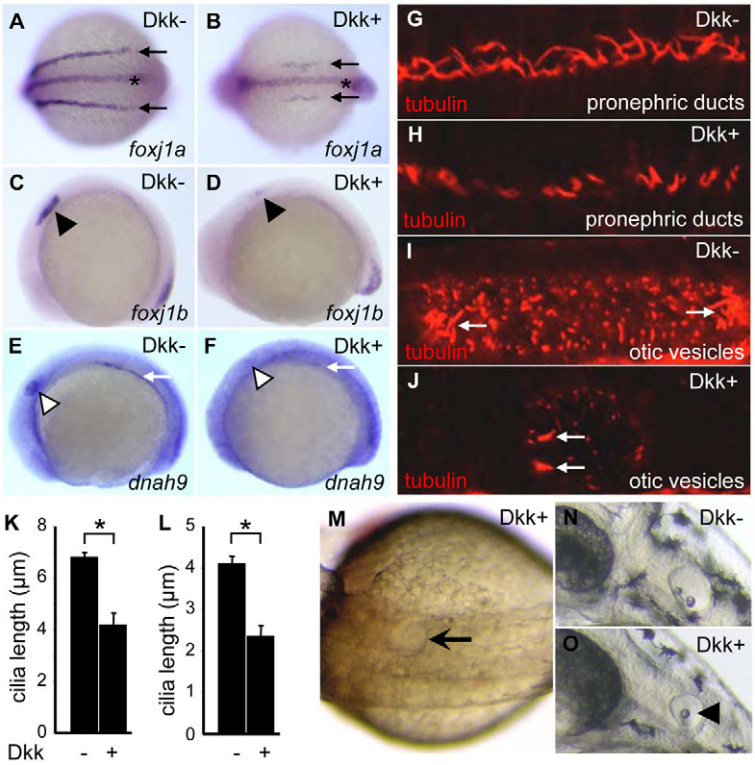

Fig. 6. Dkk1 induction impairs ciliogenesis in PD and OV. (**A-D**) Induction of Dkk1 downregulates *foxj1* expression in PDs and OVs. *foxj1a* level in PDs was downregulated in transgenic Dkk1 embryos (B; 8/8) versus non-transgenic siblings (A). *foxj1a* expression in FP was not affected (B). *foxj1b* expression in the otic placode was downregulated in transgenic Dkk1 embryos (D; 8/8) versus non-transgenic siblings (C). Arrow indicates PDs, asterisk FP, and arrowhead the otic placode. (**E**,**F**) Induction of Dkk1 suppressed *dnah9* expression in PDs and OVs (F; 11/11) compared with controls (E). White arrow represents PDs, and white arrowhead the otic placode. Shown are dorsal views (A,B) and lateral views (C-F) of 10-somite staged embryos with anterior to the left. (**G-J**) Induction of Dkk1 results in shorter and fewer cilia in PDs and OVs. Cilia were visualized by anti-tubulin antibody staining of 26-somite staged embryos. White arrow indicates tethering cilia in OVs. (**K**,**L**) Quantification of cilia length in PDs (K) and OVs (tethering cilia) (L). Data are represented as mean±s.d. Approximately 9-18 embryos were used for each group. **P*<0.01. (**M-O**) Induction of Dkk1 results in kidney cysts and otolith malformation. Cystic distension of PD (arrow in M; 45/73) was observed at 24 hpf. One otolith (arrowhead in O; 89/95) was seen at 2 dpf. *Tg(hsp:dkk1-GFP)* embryos were heat activated at 60% epiboly.

2004; El Zein et al., 2009; Cruz et al., 2010). It would be interesting to know whether Rfx3 has a similar redundant function in the node. Therefore, more experimentation is required to assess to what extent the Wnt-Foxj1-ciliogenesis cascade is conserved in mammals, and tissue- and species-specificity need to be considered.

Functional complexity between Wnt/β-catenin signaling, Wnt/PCP signaling and cilia

The present work uncovers a role of Wnt/β-catenin signaling upstream of ciliogenesis, which is distinct from studies that have focused on the effects of primary cilia on the Wnt/β-catenin pathway. The effects of cilia on the Wnt pathways have been substantially investigated, but there is a lack in consensus (Eggenschwiler and Anderson, 2007; Wallingford and Mitchell, 2011). For example, some studies suggest that cilia execute an inhibitory role on the Wnt/β-catenin pathway (Gerdes et al., 2007; Corbit et al., 2008) and are required for switching between the Wnt/β-catenin and Wnt/PCP pathways (Simons et al., 2005); others suggest a dispensable function of cilia in the Wnt pathways (Huang and Schier, 2009; Ocbina et al., 2009). Our discoveries will open doors for future studies to elaborate on a reciprocal relationship between Wnt activity and cilia formation, which might help to reconcile discrepancies in the roles of cilia in Wnt signal transduction.

The Wnt/PCP pathway has also been implicated in cilia motility. In contrast to the Wnt/β-catenin pathway that transcriptionally regulates *foxj1a*, PCP effectors and core proteins regulate cell and basal body polarization (Oishi et al., 2006; Park, T. J. et al., 2006; Park et al., 2008; Gray et al., 2009; Mitchell et al., 2009; Borovina et al., 2010; Guirao et al., 2010; Song et al., 2010). In fact, ligands and receptors that participate in these two pathways are colocalized in many ciliated tissues. Using KV as an example, genes encoding components of the Wnt/β-catenin pathway, such as *wnt3a*, *wnt8a* and *fzd10*, and genes encoding components of the Wnt/PCP pathway, such as *wnt5b* and *fzd2*, are co-expressed near or within KV (Oishi et al., 2006; Lin and Xu, 2009; Freisinger et al., 2010). Therefore, it is possible that the Wnt/β-catenin pathway governs the expression of genes that are essential for cilia formation, whereas the PCP pathway ensures that these cilia-forming components are assembled at a correctly polarized subcellular location, such as the apical surface. Further investigations are warranted to examine potential interactions between the two pathways in programming polarized cilia beating and subsequent unidirectional fluid flow.

Certain developmental functions of Wnt/β-catenin signaling might be attributed to the regulation of cilia formation

The Wnt/β-catenin pathway is an important developmental pathway that has multifaceted functions during embryogenesis. It is involved in KV, kidney and inner ear development and function (Ohyama et al., 2006; Park et al., 2007; Jayasena et al., 2008; Lancaster et al., 2009; Lin and Xu, 2009; Lyons et al., 2009). However, Wnt signaling does not appear to be essential for FP development despite the presence of Wnt ligands (Placzek and Briscoe, 2005). In keeping with these observations, we found that Wnt is required for *foxj1* expression in KV, PDs and OVs but not in FP. Our observations suggest a hypothesis predicting that defective ciliogenesis is an important cellular event that accounts for certain defects in Wnt-related developmental processes and diseases. As *foxj1* expression was associated with differentiation in tissues, such as respiratory epithelial cells, radial glia of the brain and sensory hair cells of the inner ear (You et al., 2004; Park, K. S. et al., 2006; Jacquet et al., 2009; Yu et al., 2011), it is tempting to speculate that the Wnt-Foxj1 axis represents a differentiation pathway that drives the committed epithelium towards more terminally differentiated cells with cilia. Whether and how the

Wnt-Foxj1 axis interacts with other aspects of Wnt signaling, such as cell-fate stabilization and cell proliferation, remains to be determined. Nevertheless, our findings assigned a novel function to Wnt/β-catenin signaling, which could have a significant impact on subsequent research areas that bridge cilia biology and developmental signaling.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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