Both ciliary and non-ciliary functions of Foxj1a confer Wnt/β-catenin signaling in zebrafish left-right patterning

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ABSTRACT

The Wnt/β-catenin pathway is implicated in left-right (LR) axis determination; however, the underlying mechanism remains elusive. Prompted by our recent discovery that Wnt signaling regulates ciliogenesis in the zebrafish Kupffer’s vesicle (KV) via Foxj1a, a ciliogenic transcription factor, we decided to elucidate functions of Foxj1a in Wnt-regulated LR pattern formation. We showed that targeted injection of wnt8a mRNA into a single cell at the 128-cell stage is sufficient to induce ectopic foxj1a expression and ectopic cilia. By interrogating the transcription circuit of foxj1a regulation, we found that both Lef1 and Tcf7 bind to a consensus element in the foxj1a promoter region. Depletion of Lef1 and Tcf7 inhibits foxj1a transcription in the dorsal forerunner cells, downregulates cilium length and number in KV, and randomizes LR asymmetry. Targeted overexpression of a constitutively active form of Lef1 also induced an ectopic protrusion that contains ectopic transcripts for sox17, foxj1a, and charon, and ectopic monocilia. Further genetic studies using this ectopic expression platform revealed two distinct functions of Foxj1a; mediating Wnt-governed monocilia length elongation as well as charon transcription. The novel Foxj1a-charon regulation is conserved in KV, and importantly, it is independent of the canonical role of Foxj1a in the biosynthesis of motile cilia. Together with the known function of motile cilia movement in generating asymmetric expression of charon, our data put forward a hypothesis that Foxj1a confers both ciliary and non-ciliary functions of Wnt signaling, which converge on charon to regulate LR pattern formation.

KEY WORDS: Charon, Ciliogenesis, Foxj1a, Wnt/β-catenin signaling

INTRODUCTION

Vertebrates display a distinct left-right (LR) asymmetry in the disposition of their internal organs (Raya and Belmonte, 2006). The specification of LR axis can be divided into at least two stages: (1) the generation of asymmetric signals around the mouse embryonic node or its derivatives, such as the zebrafish Kupffer’s vesicle (KV), and (2) transferring of the asymmetric cues to the lateral plate mesoderm (LPM) and internal organs (Nonaka et al., 2002; Essner et al., 2005; Kramer-Zucker et al., 2005; Hirokawa et al., 2006). Motile cilia have been shown to play a crucial role in establishing LR asymmetry at the first stage. Motile cilia occur as a single cilium extending from cells in the node or KV, where coordinated cilia beating generates a leftward fluid flow that is essential for generating asymmetric signals around the node, such as left-side downregulation of Cerberus/Dan family members Coco (Xenopus), Cerl2 (mouse), and charon (medaka and zebrafish) (Hojo et al., 2007; Oki et al., 2009; Schneider et al., 2010; Schweickert et al., 2010; Nakamura et al., 2012). The asymmetric expression of these Nodal antagonists promotes Nodal (Spaw in zebrafish) activity on the left side of the node, which is then transferred and propagated to the left LPM (Kawasaki et al., 2011).

The Wnt/β-catenin pathway has been shown to play a role in regulating LR pattern formation. Wnt activation by KV-specific overexpression of stabilized β-catenin or KV-specific depletion of Axin, a Wnt/β-catenin antagonist, results in randomized side-specific gene expression (Schneider et al., 2008), whereas global Wnt activation at levels not causing severe embryo malformation affects the competence of heart field and gives rise to no-looping heart without appreciably altering asymmetric gene expression in LPM (Carl et al., 2007; Lin and Xu, 2009). In contrast, loss of function of Wnt leads to randomized side-specific gene expression and randomized organ laterality as noted in mouse Wnt3a mutant, as well as zebrafish wnt3a, wnt8a, β-catenin and fzd10 morphants (Nakaya et al., 2005; Lin and Xu, 2009; Caron et al., 2012; Zhang et al., 2012).

At the zebrafish LR organ KV, we and others showed that inhibition of Wnt signaling results in shorter and fewer cilia, disordered fluid flow, downregulation of charon, and reduced cell proliferation (Lin and Xu, 2009; Caron et al., 2012; Zhang et al., 2012). Further investigation suggests that Wnt/β-catenin signaling regulates ciliogenesis via transcriptional control of foxj1a (Caron et al., 2012), a forkhead domain-containing transcription factor that is necessary for ciliogenesis in multiciliated cells of the mouse airway epithelial cells and monocilia biosynthesis in the zebrafish KV and Xenopus gastrocoel roof plate (GRP, frog equivalent of mouse node) (Chen et al., 1998; Brody et al., 2000; Stubbs et al., 2008; Yu et al., 2008). Consistent with Wnt-foxj1 regulation, a recent study in Xenopus reported expansion of foxj1 expression domain in the GRP by ectopic expression of β-catenin (Walentek et al., 2012). However, the Wnt-Foxj1-ciliogenesis-LR asymmetry hypothesis is not completely compatible with observations in the mouse. It has been shown that Wnt3a deficiency is associated with lack of coexpression of mechanosensing proteins PC1 and PC2 in the cilium without affecting cilium structure and motility in the node (Nakaya et al., 2005). While Foxj1 is expressed in the mouse node and deletion of the gene results in randomized LR asymmetry as Foxj1a does in zebrafish, nodal cilia are present in the Foxj1 knockout mice (Chen et al., 1998; Brody et al., 2000; Stubbs et al., 2008; Yu et al., 2008). Together, these inconsistencies suggest other, unrecognized functions of Foxj1 in LR pattern formation, prompting the present study to further interrogate functions of the Wnt-Foxj1 signaling axis in LR patterning.
Here, we present biochemical and genetic evidence to indicate that Wnt signaling directly regulates foxj1a transcription in KV through cooperative action of Lef1 and Tcf7. Using a targeted overexpression platform, i.e. injection of mRNAs into a single cell at the 128-cell stage (Agathon et al., 2003), we showed that Wnt activation induces ectopic foxj1a expression and ectopic cilia formation, possibly secondary to ectopic KV development. We revealed two distinct roles of Foxj1a in conferring Wnt-governed LR patterning. While Wnt controls cilia outgrowth via the canonical role of Foxj1a in ciliogenesis, it regulates charon expression via a novel non-ciliary function of Foxj1a.

RESULTS

Wnt activation promotes foxj1a transcription and induces ectopic foxj1a and ectopic cilia

Given that Wnt/β-catenin signaling is required for foxj1a expression and ciliogenesis (Caron et al., 2012), we set out to test the effect of gain-of-Wnt-function. Our previous studies showed a transient activation of foxj1a in the zebrafish dorsal forerunner cells (DFCs) by inducible expression of β-catenin1, although steady-state expression of foxj1a was not altered by overexpression of Wnt3a, Wnt8a, and β-catenin1 (Caron et al., 2012). To validate the transient activation, we used an inducible transgenic Tg(hsp:wnt3a-GFP) strain. The foxj1a transcript level was increased at 1 h after Wnt3a induction (Fig. 1A,B), but returned to a level comparable to that of wild-type embryos at 4 h after heat shock (Fig. 1C,D). In contrast to causing transient upregulation of foxj1a in DFCs, Wnt3a and β-catenin1 activation was found to continuously increase foxj1a expression in the developing pronephros, another tissue in which Wnt is required for foxj1a expression (Fig. S1A-D; data not shown) (Caron et al., 2012).

To seek additional evidence to support a positive regulation of Wnt signaling on foxj1a transcription, we adopted a targeted injection strategy, i.e. injection of mRNAs into a single cell at the 128-cell stage, which allows important developmental pathways to be strongly activated without serious disruption of general development (Fig. 1E) (Agathon et al., 2003). Consistent with our previous report, a tailbud-like protrusion was induced in 20.3% (48/241) of wnt8a mRNA-injected embryos (Fig. 1F) (Lin et al., 2007). Importantly, all protrusions that were examined expressed ectopic foxj1a (Fig. 1G). In addition, they contained ectopic cilialike structure as revealed by acetylated α-tubulin immunostaining (Fig. 1H). Together, these data suggest that Wnt activation induces foxj1a expression and ectopic cilia formation.

Lef1 and Tcf7 cooperatively regulate foxj1a expression and ciliogenesis in KV via binding to the foxj1a regulatory sequence

To further elucidate the Wnt-Foxj1a signaling axis, we endeavored to define the transcription circuits that regulate foxj1a expression. Among five members of the Lef/Tcf family that have been identified in zebrafish (lef1, tcf7, tcf7l1a, tcf7l1b, and tcf7l2) (Dorsky et al., 1999; Kim et al., 2000; Young et al., 2002; Veien et al., 2005), lef1 and tcf7 transcripts were detected in DFCs (Fig. S2A) and near or within KV (Caron et al., 2012). We then performed a chromatin immunoprecipitation assay to test for a physical interaction between Lef1 and a 0.6-kb foxj1a regulatory sequence, which harbors three putative Lef/Tcf binding sites (D1, D2, and D3) and recapitulates the expression pattern (Fig. 2A) (Caron et al., 2012). Lef1 coprecipitated with a distal ~0.2-kb foxj1a fragment containing D1 site and a proximal ~0.2-kb fragment containing D2 and D3 sites (Fig. S2B). To validate this association as direct protein-DNA interaction, electrophoretic mobility shift assay was performed. A Lef1-GST fusion protein, but not GST alone, bound efficiently to the 0.6-kb foxj1a fragment, and this binding could be specifically outcompeted with excess cold probe (Fig. 2B). Moreover, Lef1 bound predominantly to the D2 sequence but failed to interact with a mutated D2 oligonucleotide that disrupts the consensus Lef1 recognition sequence (Fig. 2C). Similarly, Tcf7 also bound predominantly to the D2 sequence (Fig. S2C). These biochemical studies suggest that Lef1 and Tcf7 directly bind to the same consensus site of the foxj1a promoter region.

Fig. 1. The effect of Wnt activation on foxj1a expression and cilia formation. (A-D) Wnt3a induction transiently enhances foxj1a levels in DFCs. Tg(hsp:wnt3a-GFP) embryos were heat activated at 30% epiboly for 30 min, and GFP+ (Wnt3a+) embryos were selected under fluorescence microscope. foxj1a transcript levels were examined at 1 h (A,B) and 4 h (C,D) after heat shock. Shown are dorsal views of embryos at 50% epiboly (A,B) and 95% epiboly (C,D). (E-H) Targeted overexpression of Wnt8a results in ectopic foxj1a expression and ectopic cilia formation. Schematic diagram shows a single cell injection at the 128-cell stage (E). Injection of wnt8a mRNA (100 pg) into a single cell at the 128-cell stage induced an ectopic protrusion (arrow in F), ectopic foxj1a expression (arrow in G, 8/8), and ectopic cilia-like structures as manifested by immunostaining using an antibody against acetylated α-tubulin (H). Whole-mount immunostaining was performed first, and then the protrusion was removed and imaged. Scale bar: 20 µm.
were loaded onto a 4% acrylamide non-denaturing gel. (C) Lef1 predominantly presence or absence of 50-fold excess of cold probe. The resulting products

of offspring from a double heterozygous

approximately

incubated with a32P-labeled 0.6-kb

protein (100 ng) or glutathione S-transferase (GST) alone (100 ng) was

enhancer by electrophoretic mobility shift assay. Lef1-GST fusion

foxj1a

and D3 represent putative Lef1/Tcf binding sites. (B) Lef1 interacts with the

of a 0.6 kb

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motif, serving as a positive control for Lef1 binding.

Fig. 2. Lef1 binds to the

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sequence. (A) Schematic diagram

of approximately −5.2 kb to −4.6 kb upstream of the ATG start codon. D1, D2, and D3 represent putative Lef1/Tcf binding sites. (B) Lef1 interacts with the

foxj1a enhancer by electrophoretic mobility shift assay. Lef1-GST fusion

protein (100 ng) or glutathione S-transferase (GST) alone (100 ng) was

incubated with a32P-labeled 0.6-kb foxj1a regulatory sequence in the

presence or absence of 50-fold excess of cold probe. The resulting products

were loaded onto a 4% acrylamide non-denaturing gel. (C) Lef1 predominantly binds to the D2 site. A Lef1-GST fusion protein (100 ng) was incubated with

32P-labeled oligonucleotides corresponding to D1, D2, D3, and mutated D2

(D2m) sites in the presence or absence of 50-fold excess of unlabeled oligonucleotide. The reaction mixtures were loaded into a 6% acrylamide non-

denaturing gel. TCR indicates T-cell antigen receptor enhancer AAGTTTC

motif, serving as a positive control for Lef1 binding.

Next, we conducted genetic studies to assess the role of Lef1 and Tcf7 in foxj1a expression in DFCs and ciliogenesis in KV by employing mutant

lef1nl2/+/ and tcf75kbkg21cEt/+ zebrafish (Nagayoshi et al., 2008; McGraw et al., 2011). While a moderate reduction in foxj1a transcript level was observed in 23.9% (32/134) of offspring from a heterozygous lef1nl2/+ incross or 20% (26/130) from a heterozygous tcf75kbkg21cEt/+ incross, further reduction of foxj1a transcript levels was noted in 6.3% (21/331) of offspring from a double heterozygous lef1nl2/−/−; tcf75kbkg21cEt/+ incross. Genotyping analysis confirmed that homozygous lef1, tcf7, and lef1/tcf7 mutants have reduced foxj1a expression in DFCs (Fig. 3A-D). Homozygous lef1 mutants had shorter KV cilia (4.61±0.76 μm vs 5.30±0.59 μm in wild-type siblings, P=0.012) but maintained normal cilia number (41±16 relative to 41±10, P=0.91) (Fig. 3F,M,N). Cilia in homozygous tcf7 embryos were similar to those in wild type (length 4.86±0.44 μm, P=0.09; number 39±15, P=0.52) (Fig. 3G, M,N). Cilia in the double homozygous lef1;tcf7 mutants were significantly shorter (4.22±0.38 μm, P=1.94×10−5) and fewer (30±12, P=0.009). Decrease in cilia number is less likely due to a reduction in DFCs because sox17 expression was not altered (Fig. S3B).

We then asked whether Lef1 and Tcf7 regulate foxj1a expression

KV cell-autonomously. DFC-targeted injection of lef1 MO inhibited foxj1a transcript level (Fig. S3A) and significantly reduced cilia length (3.08±0.51 μm vs 4.37±0.64 μm in controls, P=6.54×10−4) and number (23±6 vs 39±10, P=5.21×10−6) (Fig. 3I,J,O,P). DFCtcf7 MO embryos exhibited similar decreases in foxj1a expression (Fig. S3A) and ciliogenesis (length 2.99±0.81 μm, P=1.56×10−6; number 22±12, P=9.03×10−4). Further reduction in cilia length (2.21±0.55 μm) but not numbers (18±8) was observed in DFClef1tcf7 MO embryos (Fig. 3L,O,P). Comparing with mutants, lef1 or tcf7 morphants had shorter and fewer cilia, even when normalized by KV size (data not shown).

We attributed the more severe defects to a more complete disruption of Lef1 or Tcf7 function by MO injection. Indeed, the lef1 MO targets a splice donor site at exon7/intron7, which is more upstream than the point mutation in the last exon of the lef1m2 allele (Ishitani et al., 2005; McGraw et al., 2011); the tcf7−ATG MO is expected to disrupt both maternal and zygotic mRNA translation, while maternal tcf7 mRNA remains functional in the tcf7 mutants (Nagayoshi et al., 2008). Consistent with KV cilia defects, LR patterning, as revealed by left-side-specific spaw expression in LPM, was cooperatively disrupted by knocking down lef1 or Tcf7, or both [DFClef1 MO embryos: 58% left, 21% right, 21% bilateral, 4% absence (n=72); DFCtcf7 MO embryos: 63% left, 16% right, 21% bilateral (n=57); DFClef1 MO+tcf7 MO embryos: 30% left, 40% right, 20% bilateral, 10% absence (n=60)] (Fig. 3Q). Collectively, these genetic results suggest that Lef1 and Tcf7 cooperatively regulate foxj1a expression, KV ciliogenesis, and LR axis determination in a KV cell-autonomous manner.

To validate the role of Lef1 in mediating Wnt in ciliogenesis, we overexpressed Lef1 in embryos depleted of Fzd10, a proven receptor for Wnt/β-catenin signaling (Caron et al., 2012). Because Lef1 lacks transactivation activity, a constitutively active form of Lef1 (CALef1) was generated. Injection of CALef1 mRNA alone into DFCs did not significantly affect steady mRNA levels, significantly reduced cilia length (2.21±0.55 μm) but not numbers (23±6 vs 39±10, P=0.012) in DFCtcf7 MO embryos to 72%, a level comparable to that of CALef1 MO embryos (30±12, P=0.02) in DFCtcf7 MO embryos (Fig. S4E-G,I-K, M,N). CALef1 overexpression also restored left-sided spaw expression from 45% in DFCfzd10 MO embryos to 72%, a level comparable to that of CALef1 mRNA injection alone (Fig. S4O). Consistent with other methods to activate Wnt signaling, injection of CALef1 mRNA alone into DFCs did not significantly affect steady cilia length (2.21±0.55 μm) (Fig. S4A-D), proving the functionality of the fusion protein.
Targeted overexpression of Lef1 induces ectopic DFC lineage, KV-like structure, foxj1a expression, and cilia formation

Injection of CAlef1 mRNA at the 1-cell stage resulted in ectopic foxj1a expression in a region near DFCs (18 of 146 injected embryos; Fig. 4B), while injection of CAlef1 mRNA into a single cell at the 128-cell stage induced more profound tailbud-like protrusions in 25% (72/288) of injected embryos (Figs 4D and 5A). Compared with Wnt8a-induced ectopic structures, these protrusions are more visually identifiable, facilitating later experiments. Consistent with Wnt8a overexpression, CALef1-induced protrusions expressed ectopic foxj1a (Fig. 4D), and contained ectopic monocilia as assessed by acetylated α-tubulin antibody staining (green) and apical-basal polarity of KV cells by anti-ZO-1 staining (red). (E-L) DFC-specific depletion of Lef1 or Tcf7 results in shorter and fewer cilia. lef1 MO (2 ng) and/or tcf7 MO (2 ng) were injected into the yolk cells at the 256-cell stage, and embryos were collected at the 10-somite stage for immunostaining. (M–P) Quantification of cilia length and number in mutants (M,N; 11–32 embryos were scored for each genotype) and morphants (O, P; 11–31 embryos were analyzed for each group). Data are represented as means ± s.d. (Q) DFC-specific depletion of Lef1 or Tcf7 randomizes left-sided spaw expresion. Percentage of embryos with specific patterns of spaw expression was scored at the 21-somite stage. wt, wild type; A, absence; B, bilateral; L, left side; R, right side. *P<0.05, **P<0.01 compared with controls. Scale bars: 20 μm in E–L.
motile. Together, these data confirmed that Wnt activation is sufficient for foxj1a induction and ectopic cilia formation.

The Wnt/β-catennin pathway plays pleiotropic roles during embryonic development, including cell fate specification, cell type differentiation, stem cell maintenance, cilia regulation, and tissue morphogenesis (van Amerongen and Nusse, 2009; Caron et al., 2012; Clevers and Nusse, 2012; Zhang et al., 2012). The presence of monocilia similar to KV cilia in length prompted us to test the hypothesis that CALef1 is sufficient to induce the formation of KV-like cells in ectopic protrusions. Indeed, we detected the expression of sox17, a DFC lineage marker, and charon, a Nodal antagonist that is expressed only in the cells lining the KV in teleosts, are the first molecules exhibiting asymmetric expression in KV (Lin and Xu, 2009; Zhang et al., 2012). Additionally, we detected tight junction marker ZO-1 staining in the cilia-forming area of the protrusions (Fig. 5B), suggesting the presence of differentiated epithelial cells. Ectopic expression of sox17, charon, and ZO-1 was also observed after injection of wnt8a mRNA alone did not significantly alter charon transcription at the 1-cell stage downregulated and barely detectable (Hashimoto et al., 2004; Caron et al., 2012). The expression profile also supports that Foxj1a might function upstream of charon. Consistently, injection of foxj1a MO at the 1-cell stage downregulated charon transcript levels in KV (Fig. 6B). To evaluate the role of Foxj1a in Wnt-charon regulation, we performed foxj1a rescue experiments. While injection of foxj1a mRNA alone did not significantly alter charon transcription (Fig. 6H), it partially rescued charon levels in DFC6ad10 MO embryos (Fig. 6C,D) and Dkk1-expressing embryos (Fig. 6F,G). The epistatic analysis strongly suggests that Foxj1a mediates Wnt-regulated charon expression.

**Fig. 4. Targeted overexpression of CALef1 induces ectopic foxj1a and ectopic Cilia.** (A, B) CALef1 mRNA (100 pg) was injected into 1-cell staged embryos. Ectopic foxj1a expression was observed (arrow in B). (C-H) CALef1 mRNA (100 pg) was injected into a single cell at the 128-cell stage of embryos. Embryos with an induced ectopic protrusion were collected at 10 to 14 somites (D-G) and 21 somites (H). The ectopic protrusion contained ectopic expression of foxt1a (arrow in D) and dnah9 (arrow in H), and ectopic structures positive for anti-acetylated α-tubulin antibody staining (E, indicated by a dotted box, 5× magnification; amplified in F, 20× magnification). α-tubulin staining (green) was adjacent to γ-tubulin staining (red) (G). Shown are images taken from dissected and flattened protrusions (E,F) and JB-4 sections of the protrusions (G). Scale bars: 20 μm in F and 10 μm in G.

**Foxj1a confers Wnt-governed cilia formation and charon expression**

The robust CALef1-induced protrusion provides an experimental platform to dissect which aspects of Wnt functions are conferred by Foxj1a. Injection of foxj1a mRNA into a single cell at the 128-cell stage failed to induce ectopic protrusions, sox17 expression, and charon expression despite its ability to elicit cilia formation (Fig. S6; data not shown) (Yu et al., 2008), suggesting that not all functions of Wnt/β-catennin signaling are conferred by Foxj1a. In embryos coinjected with CALef1 mRNA and foxj1a MO, knockdown of Foxj1a had no effect on the CALef1-induced ectopic protrusion (Fig. S5F), ectopic sox17 expression (Fig. S5H), and ectopic ZO-1 expression (Fig. S5G). We also checked tailbud markers, such as ntl, that can be observed in Wnt-induced protrusions (Lin et al., 2007). Consistently, CALef1 activated ectopic ntl expression (Fig. 5E), which was not affected by Foxj1a knockdown (Fig. 5J). In contrast, we found that ectopic cilia were missing or shorter (1.37±0.26 μm, Fig. 5G) and ectopic charon expression was abolished on Foxj1a knockdown (Fig. 5I).

The abolished charon expression led us to speculate that Foxj1a might confer functions of the Wnt/β-catenin pathway in regulating charon expression in KV (Lin and Xu, 2009; Zhang et al., 2012). During embryogenesis, charon transcription is initiated at the 6-somite stage when foxj1a transcript levels in KV cells are already downregulated and barely detectable (Hashimoto et al., 2004; Caron et al., 2012). The expression profile also supports that Foxj1a might function upstream of charon. Consistently, injection of foxj1a MO at the 1-cell stage downregulated charon transcript levels in KV (Fig. 6B). To evaluate the role of Foxj1a in Wnt-charon regulation, we performed foxj1a rescue experiments. While injection of foxj1a mRNA alone did not significantly alter charon transcription (Fig. 6H), it partially rescued charon levels in DFC6ad10 MO embryos (Fig. 6C,D) and Dkk1-expressing embryos (Fig. 6F,G). The epistatic analysis strongly suggests that Foxj1a mediates Wnt-regulated charon expression.

The role of Foxj1a in charon expression is independent of its ciliogenic function

Members of Cerberus/Dan family Nodal antagonists, including charon in teleosts, are the first molecules exhibiting asymmetric
expression around the node. The interplay between these Nodal inhibitors and Nodal has been shown to provide the signals that lead to the establishment of laterality (Hojo et al., 2007; Oki et al., 2009; Schneider et al., 2010; Schweickert et al., 2010; Kawasaki et al., 2011). While their asymmetric expression is thought to be generated by directional cilia-driven fluid flow and further enhanced by asymmetric posttranscriptional decay of mRNA, the transcription of charon seems not dependent on cilia motility (Gourronc et al., 2007; Nakamura et al., 2012). To clarify the relationship among Foxj1a, cilia motility, and charon transcription, we inspected the following mutants with defective motile cilia: oval (fit9/8^S006/8^S006) whose KV cilia are shorter or missing and cilia beating is barely detectable, lok (ccdc40^20237b/20237b) that have reduced cilia length and motility, and ntl^6i95/6i95 that harbor severely shorter or missing cilia in KV (Kramer-Zucker et al., 2005; Amack et al., 2007; Sullivan-Brown et al., 2008; Becker-Heck et al., 2011). oval and lok mutants exhibited normal foxj1a expression and, consequently, normal charon transcript level (Fig. S7B,D,F,H). In contrast, ntl mutants exhibited loss of foxj1a and, conversely, loss of charon (Fig. S7J,N). The lack of foxj1a and charon expression was not due to absence of DFCs because sox17 was still expressed (Fig. S7L). Importantly, ectopic expression of Foxj1a rescued charon levels in ntl mutants (Fig. S7O). Taken together, these correlated data support that Foxj1a determines charon transcription while cilia beating initiates its asymmetric expression around KV.

Foxj1 has been considered a master transcription factor for motile cilia biosynthesis; it also induces genes that are required for ciliary differentiation and cilia beating (Chen et al., 1998; Stubbs et al., 2008; Yu et al., 2008; Choksi et al., 2014). Judging from the time course of foxj1a and charon expression in DFCs and KV, charon is less likely a direct transcriptional target of Foxj1a. To test this possibility and to discern roles of Foxj1a in ciliary gene induction and charon expression, we overexpressed DNA-binding domain of Foxj1a (DBD-Foxj1a) (Hackett et al., 1995). DBD-Foxj1a is expected to compete with endogenous Foxj1a for binding to the promoter region of Foxj1a-inducible genes, thus acting as a dominant negative form of Foxj1a via abolishing its transcriptional activity. Meanwhile, endogenous Foxj1a remains intact and presumably still executes other functions independent of its transcriptional activity. We found that injection of 2 ng of DBD-Foxj1a mRNA resulted in downregulation of dnah9 and efhc1, two Foxj1a target genes that are structural and functional components of the ciliary apparatus (Fig. 7B,E) (Stubbs et al., 2008; Yu et al., 2008; Choksi et al., 2014). As a consequence, KV cilia were significantly shorter (3.96±0.21 μm compared with 5.05±0.35 μm in wild-type embryos, P<8.37×10^-4) and marginally fewer (31±10 vs 38±14, P=0.20) (Fig. 7K,M,N), and left-sided spaw expression was randomized (64% left, 13% right, 21% bilateral, 2% absence [n=128]) (Fig. 7O). Injection of a higher amount of DBD-Foxj1a mRNA (10 ng) abolished dnah9 and efhc1 expression, further reduced cilia length (3.20±0.28 μm, P=2.88×10^-7) and number (25±7, P=4.97×10^-3), and enhanced abnormal spaw expression [49% left, 18% right, 29% bilateral, 4% absence (n=108)] (Fig. 7C,F,L-O). In contrast, charon expression levels remained unaffected by DBD-Foxj1a overexpression (Fig. 7H,I), indicating that Foxj1a-regulated charon expression is independent of the canonical role of Foxj1a as a ciliogenic transcription factor.

**DISCUSSION**

As described in this article, we revealed the transcription circuit of foxj1a regulation and validated our previous finding that Foxj1a is a direct transcriptional target of Wnt (Caron et al., 2012). We further uncovered two critical roles that the Wnt-Foxj1a axis plays during LR pattern formation. First, Wnt regulates cilia growth via a ciliary function of Foxj1a. This is a known function of Foxj1a that is...
dependent on its ciliogenic function. Second, Wnt determines charon transcription via a non-ciliary function of Foxj1a. This is a novel function of Foxj1a that is independent of its canonical role as a ciliogenic transcription factor. Because cilia defects will affect directional nodal flow and subsequent asymmetric expression of charon, we propose a model that ciliary and non-ciliary functions of Wnt-Foxj1a signaling converge at charon to regulate LR axis determination (Fig. 8). Our novel discovery of non-ciliary function of Foxj1a underlying Wnt-implicated laterality defect adds new knowledge to the field of LR asymmetry and might be applicable to other signaling pathways that regulate Foxj1a expression.

**Molecular nature of Wnt/β-catenin signaling in foxj1a activation**

We presented both biochemical and genetic evidence to suggest that Lef1 and Tcf7 are transcription factors that cooperatively confer Wnt-regulated foxj1a transcription during DV development. Unlike severe disruption by inhibition of Wnt signaling (Lin and Xu, 2009; Caron et al., 2012), lef1 and/or tcf7 mutants exhibit moderate defects in foxj1a expression, ciliogenesis, and presumably, LR asymmetry. This phenomenon is likely attributable to maternal expression of Lef1 and/or Tcf7, which compensates for loss of zygotic Lef1 and/or Tcf7 in the mutants (Dorsky et al., 1999; Veien et al., 2005). Precedent for this scenario comes from the observation that cilia can still form in zygotic oval mutants but are absent in maternal-zygotic oval mutants during early embryogenesis (Huang and Schier, 2009). Implication of other Tcf5 in KV ciliogenesis is less likely because we were unable to detect expression of other tcf5 in DFCs or KV.

Besides Lef1 and Tcf7, several observations prompted us to propose that Wnt needs cofactors to fulfill its function on foxj1a expression. First, Wnt signaling has a broader functional domain than foxj1a, which exhibits a more tissue-restricted expression pattern (Aamir and Dawid, 2008; Yu et al., 2008). Second, Wnt activation enhances foxj1a levels transiently in DFCs but continuously in the developing pronephros. It is possible that a negative feedback mechanism is utilized in DFCs while a positive feed-forward mechanism is engaged in the pronephros. Third, Wnt activation induces ectopic foxj1a expression in a small proportion of cells, which might be fate-specified to express certain transcription cofactors. In support of our cofactor hypothesis, it has been reported that members of the Lef/Tcf family bind to target DNA weakly and with moderate specificity, which demands cooperative interactions with other factors to achieve tight and specific control of target gene regulation. For example, SMADs and members of the homeodomain family (Pitx2, Cdx-1, Nrp) are engaged in cooperative interactions with Lef/Tcf5 to regulate transcription of target genes (Labbe et al., 2000; Nishita et al., 2000; Beland et al., 2004; Ishitani et al., 2005; Vadlamudi et al., 2005). Identification of Lef1 and Tcf7 laid the foundation for our future search for Wnt cofactors in controlling foxj1a expression in KV, which will enable us to gain further insight into the molecular nature of this novel function of Wnt.

**Ciliary function of Foxj1a in mediating Wnt signaling**

Overexpression of Foxj1 was shown to induce node-like monolalia in Xenopus and zebrafish by activating a large number of genes that encode components unique to motile cilia, including dynein arms, central pair, and radial spokes (Stubbs et al., 2008; Yu et al., 2008). Consistently, our data indicate that Foxj1a is responsible for cilia length elongation and cilia component expression. By employing a targeted Wnt activation system, we showed that Wnt is sufficient to induce a distinctive ectopic tissue protrusion, which can be used to conduct gain-of-Wnt-function analysis. Ectopic expression of foxj1a was detected, and knocking down Foxj1a ablated the de novo cilia synthesis, underscoring the ciliary function of Wnt-Foxj1a signaling. This platform overcomes the shortcoming of early embryonic lethality resulting from global manipulation of important developmental pathways, and is ideal for future studies to identify pathways or molecules that are involved in Foxj1a expression and cilia synthesis. In addition to cilia growth, this platform is also useful for testing candidate genes that are implicated in cilia assembly. Given that the ectopic protrusion is morphologically distinguishable, de novo synthesized cilia can be identified for structural analysis via transmission electron microscopy.

Our past and present studies placed Wnt/β-catenin signaling upstream of ciliogenesis via direct transcriptional regulation of foxj1a (Caron et al., 2012). In contrast, functions of cilia upstream of Wnt signaling are contradictorily reported. Some studies suggest that cilia exert 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). However, other investigators argue against a function of cilia in regulating Wnt signaling (Huang and Schier, 2009; Ocbina et al., 2009). Of note, we observed tissue-specific foxj1a activation by Wnt, as evidenced by transient and persistent increase in DFCs and the developing pronephros, respectively. It remains to be determined whether foxj1a levels are differentially regulated by a feedback or a feed-forward mechanism to which cilia-Wnt regulation might contribute.
Non-ciliary function of Foxj1a in transducing Wnt signaling

In addition to the canonical role of Foxj1a in ciliogenesis, our data reveal a novel function of Foxj1a in KV development, i.e. it confers Wnt-regulated charon expression. Unlike its function as a ciliogenic transcription factor, this novel function of Foxj1a is likely independent of its DNA-binding ability. Consistent with this non-ciliary function of Foxj1a, we and others found that cilia and cilia motility are not required for overall charon expression (Gourronc et al., 2007). In fact, non-ciliary functions of Foxj1 have also been suggested in other tissues, including the differentiation of radial glia.

Non-ciliary function of Foxj1a regulates charon expression.

(A-F) Overexpression of DBD-Foxj1a inhibits Foxj1a target gene expression. Injection of 2 ng of DBD-Foxj1a mRNA suppressed dnah9 (B, 8/14) and efhc1 (E, 6/14) transcript levels, and injection of 10 ng of mRNA nearly abolished dnah9 (C, 18/21) and efhc1 (F, 13/15) expression. Shown are dorsal views of 90% to 95% epiboly staged embryos. (G-I) Injection of DBD-Foxj1a mRNA did not affect charon expression at doses of either 2 ng (H) or 10 ng (I). Shown are dorsal views of tailbud region at 10 to 12 somites. (J-L) Overexpression of DBD-Foxj1a results in defective ciliogenesis. Shorter (K,L) and fewer (L) KV cilia were visualized at the 10-somite stage by anti-acetylated α-tubulin staining. Scale bar: 20 µm.

(M,N) Quantification of cilia length and number. 9 to 11 embryos were analyzed for each group. Data are represented as mean±s.d. *P<0.01 compared with controls. (O) Overexpression of DBD-Foxj1 randomizes left-sided spaw expression. Percentage of embryos with specific patterns of spaw expression was scored at the 21-somite stage. DBD, DNA-binding domain; A, absence; B, bilateral; L, left side; R, right side.

Fig. 7. Non-ciliary function of Foxj1a regulates charon expression. (A-F) Overexpression of DBD-Foxj1a inhibits Foxj1a target gene expression. Injection of 2 ng of DBD-Foxj1a mRNA suppressed dnah9 (B, 8/14) and efhc1 (E, 6/14) transcript levels, and injection of 10 ng of mRNA nearly abolished dnah9 (C, 18/21) and efhc1 (F, 13/15) expression. Shown are dorsal views of 90% to 95% epiboly staged embryos. (G-I) Injection of DBD-Foxj1a mRNA did not affect charon expression at doses of either 2 ng (H) or 10 ng (I). Shown are dorsal views of tailbud region at 10 to 12 somites. (J-L) Overexpression of DBD-Foxj1a results in defective ciliogenesis. Shorter (K,L) and fewer (L) KV cilia were visualized at the 10-somite stage by anti-acetylated α-tubulin staining. Scale bar: 20 µm.

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Figure 8. Summary of functions of Wnt-Foxj1a signaling in LR pattern formation. Wnt/β-catenin signaling regulates Foxj1a expression in KV via Lef1 and Tcf7 binding to the foxj1a regulatory sequence. The Wnt-Foxj1a axis appears to regulate LR asymmetry via at least two mechanisms: the first involves cilia outgrowth that is dependent on the canonical function of Foxj1a in cilia formation, and the second involves expression of charon that requires non-ciliary function of Foxj1a. Yellow highlighting indicates novel discoveries of the study.

Zebrafish strains

Zebrafish were maintained in accordance with the policies of the Mayo Clinic Institutional Animal Care and Use Committee. Wild-type (TL), heterozygous left1+/m2+ and tcf7+/hbg21cE1/+ lines, and transgenic Tg(hsp:dll1-GFP) and Tg(hsp:wt3a-GFP) strains were used for this work (Nagayoshi et al., 2008; Hoage et al., 2011; McGraw et al., 2011; Caron et al., 2012). Genotyping of the left1/m2+ and tcf7+/hbg21cE1/+ embryos was performed according to protocols from Alexei V. Netchiporuk’s laboratory (Oregon Health & Science University, Portland, Oregon, USA) and Koichi Kawakami’s laboratory (National Institute of Genetics, Mishima, Shizuoka, Japan), respectively.

Morpholino injections

Antisense morpholino oligonucleotides (MOs) (purchased from Gene Tools) targeting splice donor sites of left1 (left1 MO) and the translation initiation site of tcf7 (tcf7 MO), fzd10 (fzd10 MO), and foxj1a (foxj1a MO) have been described previously (Ishitani et al., 2005; Nagayoshi et al., 2008; Studts et al., 2008; Caron et al., 2012). To target MOs specifically to DFCs, MOs were injected into the yolk cell at the 256-cell stage (Amack and Yost, 2004).

Cloning and RNA injections

Full-length zebrafish left1 cDNA was amplified by an Expand High Fidelity PCR System (Roche Life Science) using 24-h-post-fertilization cDNA as a template. CALef1 was generated by fusing the carboxyl-terminal domain of zebrafish β-catenin1 (694–780 aa) to the N-terminus of zebrafish full-length Lef1 (Hsu et al., 1998; Aoki et al., 1999; Galceran et al., 2001). DBA-Foxj1a (136–240 aa) was amplified from the full-length zebrafish foxj1a cDNA (Hackett et al., 1995). The resulting cDNA fragments were cloned into pCS2+ plasmids. Capped mRNAs for left1, CALef1, DBD-Foxj1a, wt3a, foxj1a, and gfp were synthesized from their corresponding pCS2+ plasmids using the mMESSAGE mMACHINE SP6 Transcription Kit (Ambion) (Lin et al., 2007; Caron et al., 2012). To perform targeted overexpression, mRNAs (100 pg), alone or together with MOs (150 pg), were injected into single cells at the animal pole of 128-cell staged embryos. gfp mRNA (100 pg) was cojected to serve as a marker (Agathon et al., 2003; Lin et al., 2007). Embryos that harbored localized green fluorescence protein (GFP)-fluorescence signals or contained ectopic protrusions were collected at 10 to 21 somites for further analysis.

In situ hybridization and immunofluorescence

Single-color, whole-mount in situ hybridization was performed as previously described (Xu et al., 2002). Cilia, basal body, and apical-basal polarization of KV cells were visualized using antibodies against acetylated α-tubulin (Sigma-Aldrich), γ-tubulin (Sigma-Aldrich), and the tight junction protein ZO-1 (Invitrogen) as previously described (Lin and Xu, 2009). Cilia length and number were measured using AxioVision software (Zeiss) and analyzed by the Student t-test. For analysis of the ectopic protrusion, immunostaining was performed in whole-mount embryos, and then the ectopic protrusion was dissected and mounted in VECTASHIELD (Vector Laboratories), or the embryos were embedded in JB-4 plastic resin (Polysciences) and sectioned at 4-μm sections (Sullivan-Brown et al., 2011).


