β-Catenin 1 and β-catenin 2 play similar and distinct roles in left-right asymmetric development of zebrafish embryos

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SUMMARY
β-Catenin-mediated canonical Wnt signaling has been found to be required for left-right (LR) asymmetric development. However, the implication of endogenous β-catenin in LR development has not been demonstrated by loss-of-function studies. In zebrafish embryos, two β-catenin genes, β-catenin 1 (ctnnb1) and β-catenin 2 (ctnnb2) are maternally expressed and their zygotic expression occurs in almost all types of tissues, including Kupffer’s vesicle (KV), an essential organ that initiates LR development in teleost fish. We demonstrate here that morpholino-mediated knockdown of ctnnb1, ctnnb2, or both, in the whole embryo or specifically in dorsal forerunner cells (DFCs) interrupts normal asymmetry of the heart, liver and pancreas. Global knockdown of ctnnb2 destroys the midline physical and molecular barrier, while global knockdown of ctnnb1 impairs the formation of the midline molecular barrier. Depletion of either gene or both in DFCs/KV leads to poor KV cell proliferation, abnormal cilia formation and disordered KV fluid flow with downregulation of ntl and tbx16 expression. ctnnb1 and ctnnb2 in DFCs/KV differentially regulate the expression of charon, a Nodal antagonist, and spaw, a key Nodal gene for laterality development in zebrafish. Loss of ctnnb1 in DFCs/KV inhibits the expression of charon around KV and of spaw in the posterior lateral plate mesoderm, while ctnnb2 knockdown results in loss of spaw expression in the anterior lateral plate mesoderm with little alteration of charon expression. Taken together, our findings suggest that ctnnb1 and ctnnb2 regulate multiple processes of laterality development in zebrafish embryos through similar and distinct mechanisms.

KEY WORDS: Zebrafish, Left-right asymmetry, Wnt, β-Catenin, Nodal

INTRODUCTION
Regardless of symmetric external appearance, vertebrates have left-right (LR) asymmetric internal organs regarding their positioning and/or configurations (Raya and Belmonte, 2006). It has been accepted that leftward fluid flow within a ciliated organ during development results in the release of LR asymmetric cues into the left side of the body, which then initiates the Nodal cascade in the left lateral plate mesoderm (LPM) to control the left-sided development (Levin, 2005; Levin and Palmer, 2007; Palmer, 2004; Raya and Belmonte, 2006). In zebrafish embryos, such a ciliated organ is Kupffer’s vesicle (KV), a spherical organ that is transiently present in the tailbud during segmentation period (Essner et al., 2002; Okada et al., 2005; Lee and Anderson, 2008). The precursors of KV cells are dorsal forerunner cells (DFCs) that first arise at the leading edge of the dorsal blastoderm margin (i.e. embryonic shield) at the onset of gastrulation (Cooper and D’Amico, 1996; D’Amico and Melby et al., 1996). It has been demonstrated that loss of function of many genes in DFCs, e.g. ntl (Amack and Yost, 2004; Amack et al., 2007), lrdfr (dnah9 – Zebrafish Information Network) (Essner et al., 2005), axin1 (Schneider et al., 2008), nkd1 (Schneider et al., 2010), chordin and sox17 (Aamir and Dawid, 2010), leads to malformation of the KV and disruption of laterality.

The canonical Wnt signaling pathway, which is mediated by β-catenin, plays crucial roles in the generation of the body plan of an embryo (van Amerongen and Nusse, 2009; Logan and Nusse, 2004). Accumulating evidence indicates that this signaling pathway is also implicated in LR asymmetric development of vertebrate embryos. The first evidence arose from an overexpression study in Xenopus, which demonstrated a high frequency of cardiac left-right reversals following injection of Xwnt8 DNA into the dorsal blastomeres of Xenopus embryos (Danos and Yost, 1995). In chick embryos, ectopic Wnt8c or β-catenin in the right side of the node resulted in abnormal positioning of the heart and Wnt8c was postulated to act as a left determinant (Rodriguez-Esteban et al., 2001). Wnt3a+ mouse embryos carry multiple LR defects, including the heart, liver, lung and stomach positionings (Nakaya et al., 2005). Overexpression of constitutively active β-catenin in medaka causes cardiac laterality defects (Bajoghli et al., 2007). Dysregulation of Wnt signaling in zebrafish embryos has been found to cause LR defects (Carl et al., 2007; Caron et al., 2012; Lee et al., 2007; Lin and Xu, 2009; Schneider et al., 2010). The Wnt signaling pathway may regulate LR asymmetric development of vertebrate embryos by regulating ciliated organ formation and function (Schneider et al., 2010; Lin and Xu, 2009; Caron et al., 2012), the midline barrier formation (Danos and Yost, 1995; Lin and Xu, 2009; Nascone and Mercola, 1997) and bilateral Nodal signaling cascade (Rodriguez-Esteban et al., 2001; Carl et al., 2007).

In zebrafish, two β-catenin genes, β-catenin 1 (ctnnb1) and β-catenin 2 (ctnnb2), are maternally expressed (Bellipanni et al., 2006). A previous study demonstrates that maternal ctnnb2, but not ctnnb1, is essential for the dorsal organizer induction, whereas ctnnb1 may be implicated in suppressing neuroectoderm formation (Bellipanni et al., 2006). It remains unknown whether both ctnnb1 and ctnnb2 are involved in LR development through similar or different mechanisms. In this study, we investigated the requirement of ctnnb1 and ctnnb2 for left-right asymmetric development via morpholino-mediated knockdown of their

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expression in the whole embryo or specifically in DFCs. Our results indicate that both β-catenin genes are indispensable for KV and cilia formation, and for normal organ laterality. We also provide evidence to suggest that cttnb1 and cttnb2 have distinct roles in LR development.

MATERIALS AND METHODS

Fish maintenance

Tuebingen wild-type and Tg(sox17:GFP)::transgenic embryos (Chung and Stainier, 2008) were used in this study. Embryos were collected, cultured and staged as previously described (Westerial, 1995).

Morpholino injections

We used previously characterized morpholinos to target zebrafish cttnb1 and cttnb2: β-cat1MO, 5'-ATCAAGTCAAGCTGGTAGCCATAGA-3' (Lyman Gingerich et al., 2005); and β-cat2MO, 5'-CTTTAGCCTGAGCGACTTCCAAAC-3' [β-catenin-2 MO2 from Bellipanni et al. (Bellipanni et al., 2006)]. β-cat1MO served a control, which was identical in sequence to β-cat1MO except six mismatched nucleotides (5'-ATCAAGTCAAGCTGGTAGCCATAGA-3'), β-cat1MO or β-cat2MO was morphically injected at a dose of 20 ng unless otherwise stated. chaMO (5'-CAAAAAAGCCGCACTGGAAGATCAT-3') (Hashimoto et al., 2004), ntMO (5'-GACTTAGGAGGAGGATATTCTCGAT-3') (Nasevicius and Ekker, 2000) and txb16MO (5'-GCTTGAAGGCTCTGATGACCTGAT-3') (Bisgrove et al., 2005) were described before by others. For global knockdown, morpholinos were injected alone or in combinations into the yolk of one-cell stage embryos. For DFCs-specific knockdown, the morpholino was mixed with rhodamine (Sigma, R8881) and injected into the yolk at the 512-cell stage (2.75-3 hpf) as described before (Amack and Yost, 2004). At 60-70% epiboly stages, embryos with red fluorescence in DFCs were sorted out under a fluorescence stereo microscope and raised for further investigations.

Whole-mount in situ hybridization

Plasmid containing cmcl2 (myl7 – Zebrafish Information Network), foxa3, pitx2c, ftbl1, ftbl2, spaw, ntl, tbx16, sox17, foxa1a, dudab9, ftbl8, pdk2, cmclb, cmclb2 or charon was linearized and used for in vitro synthesis of antisense RNA in the presence of digoxigenin-UTP or fluorescein-UTP. In situ hybridization was performed essentially following a standard protocol with some minor modifications. Stained embryos were cleared in glycerol and photographed with a Leica MZ12 stereo scope using a Dage-MTI DC330 CCD camera. The staining intensity was analyzed using Image-Pro Plus 6.0 (Media Cybernetics).

Immunostaining and confocal microscopy

Embryos for immunostaining were fixed in BT fixative [4% paraformaldehyde, 0.15 mM CaCl2, 4% sucrose in 0.1 M PBS (pH 7.3)] overnight at 4°C. After being rinsed 3-5 times with PBST [0.1% Triton X-100 in 0.1 M PBS (pH 7.3)], embryos were boiled in EDTA (1 mM, pH 8.0) three times (5 minutes each time) using microwave oven, followed by blocking at room temperature for 1 hour in 10% heat-inactivated normal goat serum (NGS), 2% bovine serum albumin (BSA) and 1% DMSO in PBST. Embryos were then incubated overnight at 4°C with rabbit polyclonal anti-β-catenin antibody (1:500, Abcam), mouse monoclonal anti-acetylated-tubulin antibody (1:800, Sigma), rabbit polyclonal anti-ZO-1 antibody (1:100, Zymed), polyclonal anti-GFP antibody (1:200, Santa Cruz), rabbit anti-active caspase 3 antibody (1:200, DB Biosciences) or anti-pH3 antibody (1:150, Cell Signaling Technology). After washing with 2% BSA and 1% DMSO in PBST four times (30 minutes each), embryos were incubated overnight at 4°C with DyLight 488-conjugated AffiniPure Goat anti-rabbit IgG and DyLight 649-conjugated AffiniPure Goat anti-mouse IgG or DyLight 488-conjugated AffiniPure goat anti-mouse IgG and DyLight 649-conjugated AffiniPure goat anti-rabbit IgG (1:200, Jackson ImmunoResearch). Embryos were then washed with PBST, cleared with 70% glycerol in PBS and transferred into an anti-fade reagent (2% p-propyl gallate, 70% glycerol in PBS) and stored at 4°C for up to 2 weeks. Immunostained embryos were imaged under a Zeiss 710META laser-scanning confocal microscope with a 63x objective. A KV/cilia image was a sum of multiple focal planes (z-series), combined using ImageJ. The number of KV cells, as well as the number and length of cilia were analyzed using Image-Pro Plus 6.0. The β-catenin images were from a single focal plane.

KV fluid flow tracking

Fluorescent beads of 0.5 μm diameter (Polysciences) were injected into KVs of dechorionated embryos at the four- to five-somite stages. The embryos at the 10-somite stage were mounted in 1% low melting point agarose. The bead movements were recorded and processed using a Revolution XD Confocal microscope (ANDOR) and Image-Pro Plus 6.0, respectively.

RESULTS

Knockdown of cttnb1 or cttnb2 disrupts organ laterality in zebrafish embryos

In zebrafish embryos, two β-catenin genes, cttnb1 and cttnb2, are expressed maternally and zygotically (Bellipanni et al., 2006). We used β-cat1MO or β-cat2MO, which have been reported before (Lyman Gingerich et al., 2005; Bellipanni et al., 2006), to knock down the expression of cttnb1 or cttnb2, respectively. Injection of these morpholinos individually at the one-cell stage caused phenotypes similar to the previously reported (Bellipanni et al., 2006) (supplementary material Fig. S1). As a higher concentration within the range from 20 ng to 40 ng did not significantly worsen these phenotypes (supplementary material Fig. S1), we fixed the dose to 20 ng for subsequent injections. It was noted that β-cat1MO induced apoptosis to some extent, but its co-injection with p53MO did not lessen laterality defects (data not shown). In addition, injection of either morpholino obviously reduced endogenous β-catenin protein levels, and their co-injection further reduced β-catenin levels (supplementary material Fig. S2), further supporting the effectiveness of both morpholinos.

We first examined the expression of commonly used organ laterality markers after injections at the one-cell stage that could knock down cttnb1 or cttnb2 expression in the whole embryos. In cttnb1 or cttnb2 morphants, cardiac jogging and looping, as labeled by cmcl2 expression at 30 hpf (Fig. 1A-C) and 48 hpf (Fig. 1E-G), respectively, became randomized with the majority of embryos possessing an unjogged or unlooped heart (Fig. 1D,H). Examination of foxa3 expression at 48 hpf disclosed that the liver of cttnb1 or cttnb2 morphants was obviously randomized, although its expression in the pancreas of the morphants was absent (Fig. 11-M). We noted that the extent of laterality abnormalities in cttnb1 and cttnb2 morphants was comparable. These results suggest that cttnb1 and cttnb2 expressed in the whole embryos are equally important for development of organ laterality. As almost all of embryos co-injected with β-cat1MO and β-cat2MO at the one-cell stage deformed before 24 hpf, we could not investigate the effect of their double knockdown on organ laterality.

Both cttnb1 and cttnb2 transcripts were detected in DFCs at the 75% epiboly stage by whole-mount in situ hybridization (Fig. 2A,B). By immunostaining Tg(sox17:GFP)::transgenic embryos using an anti-β-catenin antibody, we detected nuclear β-catenin in DFCs at the 75% epiboly stage (Fig. 2C-E) and in KV cells at the 3- to 4-somite stages (Fig. 2F-H). Then we asked whether the activity of endogenous Ctnnb1 and Ctnnb2 in DFCs is required for normal development of laterality. To address this issue, β-cat1MO or/and β-cat2MO was injected into the yolk of midblastula embryos (at the 512-cell stage) to knock down their expression specifically in DFC/KV cells, as demonstrated previously (Amack and Yost, 2004). Co-injection of both morpholinos markedly
reduced the levels of β-catenin in nuclei and membrane of DFCs, whereas individual injections led to a slight reduction of nuclear β-catenin in some DFCs (supplementary material Fig. S3), suggesting an effectiveness of DFCs knockdown. In DFC-β-cat1MO or DFC-β-cat2MO embryos, the heart and liver/pancreas were all randomized in laterality (Fig. 21-K), as revealed by cmklc2 and foxa3 expression respectively. When β-cat1MO and β-cat2MO were co-injected into DFCs, the ratio of embryos with laterality defects was increased, indicative of their redundant functions. Besides, midblastula knockdown of ctnnb1 and ctnnb2 did not disrupt liver and pancreas formation (Fig. 2L).

ctnnb1 or ctnnb2 deficiency in a whole embryo ruins the midline barrier and disturbs spaw expression

In zebrafish, the spaw gene encodes a Nodal ligand and regulates organ laterality by relaying the asymmetric cues from the KV to the left LPM (Long et al., 2003). The initial expression of spaw occurs bilaterally around KV at 4- to 6-somite stages. At 10- to 12-somite stages, spaw starts to be expressed in the posterior left LPM, and thereafter this domain spreads anteriorly; the domain starts to fade at the 25-somite stage and becomes undetectable 24 hpf (Long et al., 2003). We found that knockdown of ctnnb1 at the one-cell stage caused a notorious delay of spaw expression in the immediate vicinity of KV and in the LPM, as examined at the 6- (Fig. 3A-C) and 13-somite stages (Fig. 3A’-C’). Injection of β-cat2MO at the one-cell stage also delayed spaw expression but less prominently (Fig. 3D,D’). At the 23-somite stage (about 21.5 hpf), over 90% of ctnnb1 morphants did not have detectable spaw expression in the LPM, whereas the majority of ctnnb2 morphants showed spaw expression in the LPM but in a randomized fashion (Fig. 3E-M). Subsequent examination at 23 hpf disclosed that the majority of ctnnb1 morphants were expressing spaw in the LPM but the pattern was randomized and that ctnnb2 morphants retained randomized spaw expression in the LPM (Fig. 3N). We consistently observed that a high proportion (>50%) of ctnnb1 or ctnnb2 morphants exhibited bilateral expression of spaw, even if the dose of corresponding MOs increased to 40 ng (data not shown). These results suggest that the deficiency of Ctnnb1/2 activity in a whole embryo, in particular ctnnb1 deficiency, delays the initiation of spaw expression and later on leads to its randomization in the LPM. Consistent with the altered spaw expression, the expression of the Nodal-regulated genes lefty1 in the left diencephalon and lefty2 in the heart primordium was lost in most of ctnnb1 or ctnnb2 morphants at the 23-somite stage (Fig. 3M) and resumed but randomized at 23 hpf (Fig. 3N).

The embryonic midline structure, comprising the notochord and the floorplate, has been considered as a barrier to prevent (Nodal) signals in the left LPM from diffusing into the right LPM (Bisgrove et al., 1999; Bisgrove et al., 2000; Meno et al., 1998). So, we investigated whether injection of ctnnb1 or ctnnb2 morpholinos at the one-cell stage could interrupt the midline barrier. ctnnb1 morphants retained the notochord and floorplate with loss of ntl expression (supplementary material Figs S1, S4), but the expression of the Nodal antagonist lefty1 in the notochord was completely or almost abolished (Fig. 3V) and the expression of ntl in the notochord was interrupted in a small caudal region (Fig. 3Q). By contrast, many ctnnb2 morphants lost the notochord and floorplate with loss of ntl, lefty1 and shh expression (Fig. 3R,S,W,X; supplementary material Figs S1, S4). This implies that ctnnb2 is required for the establishment of the midline barrier structurally and molecularly, while ctnnb1 is needed primarily for the midline molecular barrier. The absence of the midline barrier in ctnnb1 or ctnnb2 morphants may contribute to bilateral expression of spaw.

ctnnb1 or ctnnb2 deficiency in DFCs differentially alters Nodal gene expression pattern

The asymmetric expression of spaw in LPM depends on the fluid flow generated by the motile cilia in the KV (Amack and Yost, 2004; Essner et al., 2005; Kramer-Zucker et al., 2005) though left determinants (laterality cues) released by the fluid flow in zebrafish embryos have not been determined at molecular level. We asked whether the Ctnnb1/2 activity in the KV also contributes to the asymmetric expression of spaw and other Nodal-regulated genes. When β-cat1MO or β-cat2MO was injected at the midblastula stage, the initiation of spaw expression was not delayed (data not shown), but its expression was randomized at the 23-somite stage (Fig. 4A-H,P). We also observed that the ratio of embryos with randomized expression of spaw did not change much when the morpholino dose was raised to 40 ng (data not shown). Double knockdown of both genes increased the ratio of embryos with bilateral spaw expression, suggesting a partial redundancy of their function (Fig. 4P). Consistent with randomization of spaw expression, the Nodal targets lefty1 (in the diencephalon), lefty2 (in the heart primordia) and pitx2 (in the posterior left LPM, Fig. 4M-O) displayed randomized expression in DFC-β-cat1MO, DFC-β-cat2MO or DFC-β-cat1;β-cat2MO embryos (Fig. 4P). Unlike one-cell stage knockdown, the DFC-specific knockdown of ctnnb1 or ctnnb2 did not disrupt the expression of the midline molecular barrier lefty1 (data not shown). To exclude the possibility that morpholinos injected at midblastula stages affect gene functions in blastula margin on LR patterning, we injected either or both of β-cat1MO and β-cat2MO into embryonic yolk at late blastula stages (dome to...
30% epiboly stages) when morpholinos inside the yolk would diffuse into blastodermal marginal cells but not DFCs. The results indicate that left-side expression of lft2 and spaw was not altered in embryos injected at late blastula stages (supplementary material Table S1). Therefore, we conclude that the Wnt/β-catenin signaling pathway in DFCs and their derivatives (KV cells) is also crucial for LR asymmetry.

Interestingly, spaw expression in DFCβ-cat1MO or DFCβ-cat2MO embryos exhibited different patterns at the 23-somite stage. In DFCβ-cat1MO embryos, spaw stripes, wherever they were, all extended to the anterior LPM, but appeared to be thinner in the posterior LPM and retained expression domains in the posterior LPM were significantly reduced, whereas the width at the anterior position was comparable with that in wild-type embryos (supplementary material Fig. S5). By contrast, spaw expression in LR-defective DFCβ-cat2MO embryos was unable to reach the anterior LPM and retained expression domains in the posterior LPM were thinner and weaker (for examples see Fig. 4F-H). In one batch of 78 DFCβ-cat2MO embryos, for example, 40 out of 52 embryos with left-sided spaw expression only retained the posterior region of spaw domain. By measuring the total length of the left-sided spaw domain of a group of embryos, we found that this length in DFCβ-cat2MO embryos was reduced by about 40% compared with that in wild-type embryos, whereas the length in DFCβ-cat1MO embryos was not significantly changed (supplementary material Fig. S6). When both genes were simultaneously knocked down in DFCs, the majority of the spaw stripes in the LPM (48/55 in the left LPM and 24/32 in the right LPM) extended to the anterior position, although these stripes were generally thinner and weaker (Fig. 4J-L; supplementary material Fig. S7). Taking these data together, we conclude that ctnnb1 and ctnnb2 regulate spaw expression in the LPM differently.

**Deficiency of ctnnb1 but not ctnnb2 downregulates charon expression around the KV**

Charon has been considered an antagonist of Nodal signals in LR patterning (Gourronc et al., 2007; Hashimoto et al., 2004; Ray et al., 2003; Wang and Yost, 2008). In zebrafish, inhibition of wnt3 and wnt8 has been found to downregulate charon expression (Lin and Xu, 2009). However, it remains unknown whether ctnnb1 and ctnnb2 differentially regulate charon expression. We examined the effects of ctnnb1 and ctnnb2 knockdown at the one-cell or midblastula stages on charon expression. When ctnnb1 was knocked down at the one-cell stage, over 90% of morphants showed a dramatic decrease of charon expression at the six-somite and 13-somite stages (Fig. 5B,I,O). By contrast, knockdown of ctnnb2 at the one-cell stage caused a much smaller proportion of embryos with a marginal decrease of charon expression (Fig. 5C,J,O). It appears that ctnnb1 and ctnnb2 regulate charon expression within and around KV distinctly.

As charon is specifically expressed in KV lumen epithelia and surrounding cells (Hashimoto et al., 2004), we performed knockdown of ctnnb1 and ctnnb2 in DFCs and investigated their effect on charon expression. Depletion of ctnnb1 in DFCs also resulted in a marked proportion of embryos with decreased charon expression (Fig. 5E,L,P), whereas the percentage of DFCβ-cat2MO embryos with a decrease of charon was changed marginally compared with the control (Fig. 5F,M,P). Furthermore, midblastula injection of β-cat2MO at a higher dose (40 ng) did not markedly increase the ratio of embryos with decreased charon expression, as
observed at the 13-somite stage (Fig. 5P). When β-cat1MO (20 ng) and β-cat2MO (20 ng) were co-injected at midblasta stages, charon expression was decreased at an extent (Fig. 5G,N) comparable with β-cat1MO single injection and the ratio of affected embryos was only slightly increased as observed at the 13-somite stage (Fig. 5P). By measuring the integrated optical density of the charon staining area and counting the number of charon-positive cell numbers, we confirmed that DFC β-cat1MO embryos showed a significant decrease of charon expression levels and had a reduced number of charon-positive cells while injection of β-cat2MO into DFCs did not produce a statistically significant effect on charon expression (supplementary material Fig. S8). These data together indicate that β-catenin signaling pathway is required for charon expression around the KV and this function is mainly mediated by ctnnb1 rather than ctnnb2.

Recently, charon expression has been found to be transiently biased on the right side of KV in zebrafish embryos from 8- to 10-somite stages (Schneider et al., 2010). We found that knockdown of either ctnnb1 or ctnnb2 in DFCs did not significantly change the ratio of embryos with a right-side biased charon expression at the six- and 13-somite stages (data not shown).

Altered spaw expression patterns in ctnnb1/2 morphants are related to charon expression

Our next question was how alterations of charon expression in ctnnb1/2 morphants could affect spaw expression pattern in the LPM. To address this issue, we performed double knockdown of charon and ctnnb2 or ctnnb1 in DFCs, and measured the length of the spaw expression stripe and the width of the spaw domain in the LPM at different positions at the 23-somite stage. We found that, unlike in DFC β-cat2MO embryos, the total length (L1) and the length (L2) between the anterior edge and the position in parallel to the myod1-positive anteriormost edge of the spaw stripe in DFC β-cat2MO+chaMO embryos were not significantly shorter than those in the wild-type embryos (supplementary material Fig. S9B). This result suggests that inhibition of charon expression in DFC β-cat2MO allows anterior propagation of Nodal signals in the LPM to activate Nodal-regulated gene expression in the anterior LPM. When chaMO and β-cat1MO were co-injected, the width of the spaw expression stripe was not significantly narrower in morphants than in the control embryos (supplementary material Fig. S9A), suggesting a rescuing effect. It is possible that loss of remnant Charon in DFC β-cat1MO embryos by chaMO injection could compensate β-cat1MO-induced reduction of Nodal signals so that spaw expression in the LPM could be recovered. Regarding laterality of spaw expression, co-knockdown of charon with ctnnb1 or ctnnb2 generally resulted in an increase of embryos with bilateral spaw expression (supplementary material Fig. S9C).

ctnnb1 and ctnnb2 deficiency in DFCs affects KV and cilia formation and KV fluid flow

The function of cilia within the KV is a key step in development of the zebrafish LR asymmetry (Bisgrove et al., 2005l; Essner et al., 2005), which involves the function of wnt3 and wnt8 (Lin and Xu, 2009). We set out to inspect KV and cilia formation in DFC β-cat1MO or DFC β-cat2MO embryos by marking KV lumen cells using anti-ZO-1 antibody and labeling cilia using anti-acetylated-tubulin antibody. Individual or simultaneous knockdown of ctnnb1

Fig. 3. Universal knockdown of ctnnb1 and ctnnb2 disturbs spaw and Nodal-regulated gene expression and impairs the midline barrier establishment. One-cell embryos were injected with indicated morpholinos. (A-D) spaw expression in the perinodal region (black arrows) and left LPM (red arrows) at the six-somite (A-D) and 13-somite (A’-D’) stages, dorsal views. Embryos were staged by counting somite numbers. In the bottom left-hand corner was the number of embryos with the representative spaw expression around KV/total number of embryos. (E-L) Representative images of spaw expression in LPM at the 23-somite stage. (M,N) Statistical data for spaw in the LPM. n1 in the diencephalon and n2 in the heart primordium at 21.5 hpf (M) and 23 hpf (N), respectively. n, observed embryo number. (O-X) The representative patterns of ntl and lft1 in the midline (notochord) at about the 19- and 16-somite stages, respectively. The ratios are indicated in the bottom left-hand corner. Scale bars: 100 µm.
and ctnnb2 dramatically reduced the size of the KV as observed at the 10-somite stage (Fig. 6A-J), although the inflated KV apparently existed. We then counted the number of cilia within the KV and measured the length of each cilium. Results indicated that in either DFCβ-cat1MO or DFCβ-cat2MO embryos the number of cilia within the KV was reduced (Fig. 6K) and those cilia were also shorter (Fig. 6L) compared with those in the control embryos. Simultaneous knockdown of both genes did not cause further reduction of cilia number and length (Fig. 6K,L). These observations suggest that Ctnnb1 and Ctnnb2 activity within DFCs and KV cells are indispensable for normal KV and cilia formation.

We then examined cilia function by observing KV flow-driven movements of the injected fluorescent beads within the KV. The beads in wild-type embryos (Fig. 6F; supplementary material Movie 1) and DFCβ-cat1MO embryos (Fig. 6G; supplementary material Movie 2) showed regular counterclockwise movement. However, in DFCβ-cat1MO (n=7/13), DFCβ-cat2MO (n=6/8) or DFCβ-cat1+2MO (n=7/9) embryos, the moving paths of the beads became disordered (Fig. 6H-J; supplementary material Movies 3-5). These results indicated that ctnnb1 and ctnnb2 are not only required for cilia formation but also for cilia-driven KV fluid flow.

ctnnb1 and ctnnb2 deficiency in DFCs inhibits KV cell proliferation

The reduction of KV size and cilia number in ctnnb1/2 morphants implies possible functions of Ctnnb1/2 activity in proliferation and/or apoptosis of KV cells. So, we examined cell proliferation by immunostaining Tg(sox17:GFP)s870 transgenic embryos, which express GFP in DFCs and KV cells (Chung and Stainier, 2008), with anti-GFP and anti-phospho-histone 3 (pH3) antibodies and counting positive cells within the KV (Fig. 7A-J). At the three- to four-somite stages at which KV lumen is just formed (Amack et al., 2007),...
uninjected, DFC$\beta$-cat1MO, DFC$\beta$-cat2MO or DFC$\beta$-cat1+2MO embryos all had 30-40 GFP-positive KV cells (Fig. 7K); the percentage of embryos possessing pH3-positive KV cells (Fig. 7L) or the average proportion of pH3-positive KV cells (Fig. 7M) was also comparable with each other among those groups. However, at the 10-somite stage, at which the KV is fully formed and functioning, the number of GFP-positive and the proportion of pH3-positive KV cells in DFC$\beta$-cat1MO, DFC$\beta$-cat2MO or DFC$\beta$-cat1+2MO embryos were much lower than in control embryos (Fig. 7K-M). These data indicate that $ctnb1/2$ deficiency does not affect proliferation of DFCs during epiboly period but inhibits KV cell proliferation after KV lumen starts to form, leading to a small KV.

We next investigated apoptosis of KV cells by immunostaining Tg(sox17:GFP)s870 transgenic embryos with anti-active caspase 3 antibody. Results showed that the fraction of caspase 3-positive KV cells in morphants were comparable with that in control embryos (Fig. 7N). It is likely that $ctnb1/2$ deficiency does not affect survival of KV cells.

DFCs-specific $ctnb1/2$ activity regulates distinct transcription factors for cilia formation

The malformation of KV cilia in $ctnb1$ and $ctnb2$ morphants prompted us to examine a set of transcription factors in morphants, which are expressed in DFCs and implicated in cilia formation. The KV fluid flow was monitored by observing movements of injected fluorescent beads. Each colored line represented the kinetic track of a single bead and the arrowhead indicates the moving direction. The moving paths in morphants were disordered. (K,L) Quantification of cilia number (K) and length (L). Error bars indicate ±s.e.m.; n, analyzed embryo number. Statistical significance: *P>0.05; **P<0.01. Student’s t-test was used.

Fig. 6. $ctnb1$ and $ctnb2$ in DFCs are required for KV and cilia formation. Embryos were injected with individual or mixed morpholinos at the 512-cell stage and visualized after immunostaining at the 10-somite stage. (A-E) KV cilia were visualized by anti-acetylated-tubulin antibody. Morphants had fewer and shorter cilia. Scale bar: 20 μm. (A'-E') KV lumen cells labeled by anti-ZO-1 antibody. The embryos were identical to those in A-E. (F-J) The KV fluid flow was monitored by observing movements of injected fluorescent beads. Each colored line represented the kinetic track of a single bead and the arrowhead indicates the moving direction. The moving paths in morphants were disordered. Error bars indicate ±s.e.m.; n, analyzed embryo number. Statistical significance: *P>0.05; **P<0.01. Student’s t-test was used.

Fig. 7. DFCs knockdown of $ctnb1$ and $ctnb2$ inhibits KV cell proliferation. Tg(sox17:GFP)s870 transgenic embryos were injected with individual or mixed morpholinos at the 512-cell stage and analyzed later, as indicated. (A-J) Confocal images of KVs at the three- to four-somite (A-E) and 10-somite stages (F-J) following immunostaining with anti-GFP and anti-pH3 antibodies. (K) KV (GFP-positive) cell number was counted at different stages. (L) The percentage of embryos with pH3-positive KV cells. (M) The average percentage of pH3-positive KV cells per embryo. The percentage was calculated for each embryo and then averaged among all observed embryos. The same batch of embryos was subjected to different analyses shown in K-M. (N) The average percentage of caspase 3-positive KV cells per embryo. The injected embryos were co-immunostained with anti-GFP and anti-active caspase 3 antibodies at different stages and observed by confocal microscopy. *P<0.05; **P<0.01; Error bars indicate ±s.e.m.; n, analyzed embryo number.
formation. sox17 and foxj1a are expressed in the zebrafish DFCs, and foxj1a functions to promote cilia formation in various tissues (Aamar and Dawid, 2008; Amack and Yost, 2004; Yu et al., 2008; Tian et al., 2009). We found that sox17 and foxj1a expression levels in DFCβ-cat1MO, DFCβ-cat2MO or DFCβ-cat1+2MO embryos at the 90% epiboly stage were comparable with those in wild-type or control MO-injected embryos (Fig. 8A-J). This suggests that Ctnnb1/2 activity in DFCs regulates KV and cilia formation independent of Sox17 and Foxj1a. The T-box transcription factors Ntl and Tbx16 are also expressed in DFCs and KV cells, and play a crucial role in KV development (Amack et al., 2007). When ctnnb1 and ctnnb2 expression in DFCs were individually or simultaneously knocked down by midblastula injection of the corresponding morpholinos, ntl and tbx16 expression in the DFCs was decreased while their expression territories in the blastodermal margin and the axial mesoderm were unaltered at the 75% epiboly stage (Fig. 8K-U).

Previous studies have demonstrated that the ciliogenic genes dnah9, ift88 and pkd2 are regulated by ntl and tbx16 during KV cilia formation in zebrafish embryos (Bisgrove et al., 2005; Essner et al., 2005). We found that depletion of ctnnb1 or ctnnb2, or both, in DFCs also led to a decrease of the expression of dnah9, ift88 and pkd2 in DFCs (supplementary material Fig. S10). Taking these data together, we conclude that Ctnnb1/2 activity in DFCs is required for cilia formation by controlling the expression of a set of transcription factors.

### DISCUSSION

In this study, we investigated the functions of ctnnb1 and ctnnb2 genes in the zebrafish LR asymmetric development by carrying out loss-of-function studies. Knockdown of either gene in the whole embryos or specifically in DFCs randomizes the heart, liver and pancreas of the zebrafish embryos. We demonstrate that both genes are required for the establishment of the midline barrier, proliferation of KV cells, and formation and function of KV cilia. But, ctnnb1 and ctnnb2 regulate the midline barrier and the Nodal antagonist charon differently in some aspects.

### Endogenous β-catenin is required for normal laterality development

The canonical Wnt signaling pathway involves a large number of proteins. In the absence of Wnt signals, β-catenin associates with APC, Axin and GSK3β to form a destruction complex and is subjected to degradation (Barker et al., 2008; Huang and He, 2008). The binding of Wnt ligands to membrane receptors activates Dishevelled protein, which inhibits β-catenin destruction complex formation and ultimately results in stabilization of cytoplasmic β-catenin and its translocation into the nucleus to regulate target gene expression. In the past few years, studies in mouse and zebrafish have demonstrated that downregulation of several Wnt pathway components, e.g. zebrafish wnt3, wnt8, axin1, apc, gsk3b and nkd1 and mouse Wnt3a, disrupts normal LR development (Carl et al., 2007; Lin and Xu, 2009; Lee et al., 2007; Schneider et al., 2010; Nakaya et al., 2005). However, it is not clear whether and how endogenous β-catenin affects LR symmetry of vertebrate embryos. In this study, we show that inhibition of ctnnb1 or ctnnb2, or both, in the zebrafish embryos leads to defective organ laterality, providing the first evidence that both genes are implicated in LR asymmetric development.

### Functions of ctnnb1 and ctnnb2 involve the establishment of the midline barrier and initiation of spaw expression

Canonical Wnt signaling occurs throughout early vertebrate embryogenesis and regulates many developmental processes, such as organizer induction and dorsal axis formation, germ layer patterning and somitogenesis (De Robertis and Kuroda, 2004; Logan and Nusse, 2004; van Amerongen and Nusse, 2009; Weaver and Kimelman, 2004). It is expected that up- or downregulation of β-catenin inside and outside DFCs/KV would cause LR asymmetric defects. We found that one-cell stage knockdown of ctnnb1 or ctnnb2 caused much higher proportions of laterality-defective embryos than midblastula stage knockdown. At the molecular level, one-cell stage knockdown delayed spaw expression and disrupted the midline molecular barrier lft1 (Fig. 3).
which was not observed in midblastula-knockdown embryos. This implies that ctnnb1 and ctnnb2 activity outside DFCs and KV can contribute to the establishment of the midline barrier and to the initiation of spaw expression.

**ctnnb1 and ctnnb2 have different roles in the establishment of the midline barrier**

Both zebrafish ctnnb1 and ctnnb2 are maternally expressed and their zygotic expression appears to be ubiquitous (Bellipanni et al., 2006). A previous study demonstrates that maternal ctnnb2 rather than ctnnb1 is essential for dorsal organizer formation during early embryogenesis, while both genes play a redundant role in suppressing neuroectoderm formation at later stages (Bellipanni et al., 2006). ctnnb2 knockdown can lead to loss of the notochord and floorplate, and consequently the Nodal antagonist lefty1 expression in the midline is eliminated. Therefore, ctnnb2 is essential for setting up a physical midline barrier for preventing Nodal signals in the left LPM from diffusing into the right LPM. By contrast, ctnnb1 knockdown inhibits lefty1 expression in the midline, while the notochord and floorplate remain in the midline, which suggests that ctnnb1 is required primarily for the establishment of the midline barrier at the molecular level.

**Ctnnb1 and Ctnnb2 activity in DFCs/KV regulates KV cell proliferation, cilia formation and KV fluid flow**

What are the roles of Ctnnb1 and Ctnnb2 expressed in DFCs/KV? DFCs/KV-specific knockdown of either gene inhibited KV cell proliferation after initial formation of KV, but had little effect on KV cell apoptosis (Fig. 7). This suggests that Ctnnb1 and Ctnnb2 activity within DFCs/KV is required for KV cell proliferation to assure a normal size of KV. We found that depletion of Ctnnb1 and Ctnnb2 activity in DFCs downregulated, but did not eliminate, ntl and tbx16 expression in DFCs (Fig. 8). We found that co-knockdown of ntl with ctnnb1 or ctnnb2 in DFCs could lead to a dismorphic KV similar to that seen in ntl single knockdown (Amack et al., 2007; Amack and Yost, 2004) (supplementary material Fig. S11), suggesting that ntl acts downstream of β-catenin. Co-knockdown of tbx16 with ctnnb1 or ctnnb2 in DFCs could also result in a smaller KV of a size similar to that seen in tbx16 single knockdown (supplementary material Fig. S12) (Amack et al., 2007) but smaller than single knockdown of β-catenin genes (compare supplementary material Fig. S12 with Fig. 6C’,D’), which also supports the idea that tbx16 is epistatic to β-catenin. Therefore, ntl and tbx16 function on KV formation is regulated partially by β-catenin.

Furthermore, ctnnb1 and ctnnb2 deficiency led to fewer and shorter KV cilia, as well as disrupted KV fluid flow (Fig. 6). Fewer cilia in KV could be largely ascribed to the lower number of KV cells, while the reduction of cilia length could be related to dysregulation of the expression of ntl and tbx16 and their downstream ciliogenic genes dnah9, ift88 and pkd2. The malfunction of KV cilia results in reduced amounts and random diffusion of laterality cues emanating from KV.

We found that ctnnb1 and ctnnb2 deficiency did not markedly affect sox17 and foxj1a expression in DFCs/KV (Fig. 8A-J). A recent report demonstrates that foxj1a expression in DFCs/KV is reduced upon overexpression of dkk1, an antagonist of Wnt ligands, or knockdown of fzd10, a receptor of Wnt ligands (Caron et al., 2012). It is possible that DFCs/KV expression of foxj1a is also regulated by noncanonical Wnt signaling that is also mediated by Fzd10 and inhibited by Dkk1. On the other hand, we cannot exclude the possibility that, in our study, residual amount of Ctnnb1/2 in the morphants are sufficient for foxj1a expression in DFCs/KV.

**ctnnb1 in KV also regulates propagation of Nodal signals by modulating charon expression**

Our results indicate that ctnnb1 in KV could regulate propagation of Nodal signals in the LPM at least in two ways. On the one hand, ctnnb1 is required for production of laterality cues by regulating KV and cilia formation and function, which is evidenced by narrower spaw domain in the middle and posterior LPM when it is knocked down (supplementary material Fig. S5). On the other hand, ctnnb1 acts to maintain the expression of the Nodal antagonist gene charon within and around KV, thereby preventing excess Nodal signals from anterior propagation. One important evidence is that co-knockdown of ctnnb1 with ctnnb2 leads to a recovery of spaw expression in the anterior LPM that is usually absent in ctnnb2 morphants (supplementary material Fig. S7). Nuclear β-catenin can associate with DNA-binding transcription factors Tcf/Lef to activate target gene transcription (MacDonald et al., 2009). We found that there are 19 putative Tcf/Lef-binding sites in 3 kb promoter region upstream of the translation start site of charon. It is likely that β-catenin and Tcf/Lef directly regulate charon transcription.

Unlike ctnnb1 knockdown, ctnnb2 knockdown alone has little effect on charon expression (Fig. 5), but causes missing of spaw expression in the anterior LPM (Fig. 4). Therefore, normal expression of ctnnb2 is essential for sustaining a sufficient amount of β-catenin for anterior propagation of Nodal signals. An interesting phenomenon is the reduction of KV size with little alteration in charon expression in ctnnb2 morphants. This could be explained by the observation that charon is expressed in KV lumen cells (a single layer) as well as adjacent cells (Hashimoto et al., 2004) and loss of Ctnnb2 in DFCs may reduce charon expression in KV lumen cells but does not affect its expression in adjacent cells.
A model for ctnnb1 and ctnnb2 functions in key steps of laterality development

We summarized our findings in Fig. 9. Ubiquitously expressed ctnnb1 is involved in the formation of the midline molecular barrier (lft1 expression domain), and ubiquitous ctnnb2 is required for the establishment of the midline barrier at both structural and molecular levels (Fig. 9A). Ctnnb1 expressed in KV and adjacent cells mainly acts to maintain charon expression to prevent excess Nodal signals from releasing into KV. Ctnnb2 in KV cells is needed for generation and propagation of Nodal signals or other laterality cues to ensure expression of Nodal genes, including spav, in the left LPM. Both ctnnb1 and ctnnb2 in DFC/KV cells are required for KV cell proliferation, cilia formation and KV fluid flow (Fig. 9B), which in turn control the generation of laterality cues.

At present, it is not known why ctnnb1 and ctnnb2 have different functions in some aspects of laterality development. It is possible that Ctnnb1 and Ctnnb2 proteins have different molecular properties or different subcellular locations, as they have been shown to have different stability (Mo et al., 2009).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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