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Distinct functions of Wnt/β-catenin signaling in KV development and cardiac asymmetry

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The Wnt/β-catenin pathway exhibits distinct and developmental stage-specific roles during cardiogenesis. However, little is known about the molecular mechanisms of Wnt/β-catenin signaling in the establishment of cardiac left-right (LR) asymmetry. Using zebrafish as an animal model, we show here that Wnt/β-catenin signaling is differentially required in cardiac LR patterning. At an early stage, during asymmetric signal generation, Wnt/β-catenin signaling is necessary for Kupffer's vesicle development and for the regulation of both heart and visceral laterality. At a later stage, during asymmetric signal propagation, excessive Wnt/β-catenin signaling inhibits the transmission of asymmetric cues from the lateral plate mesoderm (LPM) to the cardiac field but not to the developing gut; as such, it only regulates heart laterality. Molecular analysis identifies Gata4 as the downstream target of Wnt/β-catenin signaling in the cardiac field that responds to the Wnt/β-catenin signaling and regulates the competence of the heart field to express left-sided genes. In summary, our results reveal a previously unexpected role of Wnt-Gata4 signaling in the control of asymmetric signal propagation from the LPM to the cardiac field.

KEY WORDS: Left-right asymmetry, Wnt signaling, Gata4, Lefty2, Zebrafish

INTRODUCTION

Vertebrates display distinct left-right (LR) asymmetry in the disposition of internal organs. In amniotes, the heart, spleen and pancreas are located on the left, and liver on the right (Palmer, 2004). LR asymmetry is specified during early embryogenesis by complex epigenetic and genetic cascades. The initial symmetry-breaking event is probably different among species. It could be the nodal flow in the mouse, ion fluxes in the chick and zebrafish, or asymmetric gene expression in *Xenopus* (Levin, 2005; Raya and Belmonte, 2004; Raya and Belmonte, 2006). However, this initial LR information is transmitted in every species to the embryonic node or node equivalent structures, such as Kupffer's vesicle (KV) in zebrafish. The node or KV is lined by monociliated cells with motile cilia and regulates LR patterning (Essner et al., 2005; Essner et al., 2002; Kramer-Zucker et al., 2005; Nonaka et al., 1998). Once the asymmetric signal is generated in the node or KV, it is conveyed to the left lateral plate mesoderm (LPM) by establishing left-sided expression of Nodal, which sequentially propagates the signal to organ primordia. During this process, Nodal is believed to play an instructive role to induce side-specific gene expression in organ primordia, including Nodal antagonists Lefty1 and Lefty2 in the left cardiac field and the paired-like homeodomain transcription factor Pitx2 in the left posterior LPM (Campione et al., 1999; Essner et al., 2000; Ryan et al., 1998). Although Nodal induces the expression of both Lefty2 and Pitx2 through asymmetric enhancers (ASE) (Adachi et al., 1999; Saijoh et al., 1999; Shiratori et al., 2001), the expression of Nodal and Lefty2 or Pitx2 is not always correlated. Lefty2 expression can be abolished in the presence of Nodal expression (Chocron et al., 2007; Shu et al., 2007), or ectopically induced in the absence of Nodal expression; for example, in Furin-deficient mice and in Hnf3β$^{-/-}$ aggregation chimaeras (Constam and Robertson, 2000; Dufort et al., 1998). Pitx2 expression can also be initiated in the absence of Nodal; for example, in embryos with reduced Notch activity (Krebs et al., 2003; Raya et al., 2003). Thus, both Lefty2 and Pitx2 expression might be regulated by other signaling pathways, in addition to Nodal.

Wnt/β-catenin signaling regulates multiple steps of cardiogenesis (Foley and Mercola, 2004; Tzahor, 2007). Activating the Wnt/β-catenin pathway induces lateral mesoderm formation (Ueno et al., 2007); at a later developmental stage, repressing this pathway defines the heart-forming field boundaries (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). Even later in development, activation of this pathway is important for cardiac cushion morphogenesis and valve formation (Hurlstone et al., 2003). Recently, Wnt/β-catenin signaling was shown to be involved in cardiac LR patterning. Whereas loss-of-function studies in mice indicate that Wnt3a is required for LR determination (Nakaya et al., 2005), gain-of-function studies generate results that are not always consistent with each other. In chick, Wnt8-c exhibits a species-specific asymmetric expression pattern and overexpression of Wnt8-c leads to defects in cardiac asymmetry (Rodriguez-Esteban et al., 2001). In *Xenopus*, overexpression of Xwnt8 results in a reversal of heart looping that is probably secondary to anterior notochord regression (Danos and Yost, 1995). In zebrafish, overactivation of Wnt/β-catenin signaling in *masterblind* (*mbl*) mutants and *gsk3β* morphants leads to a failure of heart looping (Carl et al., 2007; Lee et al., 2007). Other overexpression experiments also implicate Wnt/β-catenin signaling in cardiac laterality (Bajoghli et al., 2007; Schneider et al., 2008). Most of these studies focused on the functions of Wnt/β-catenin signaling during the development of the node (KV) or during the asymmetric expression of Nodal pathway genes in the LPM. In addition, *mbl* mutants exhibit brain asymmetry defects that are independent of LPM Nodal signaling, suggesting that Wnt/β-catenin signaling might act in an organ-specific manner (Carl et al., 2007). In contrast to in brain, it remains unclear whether Wnt plays any roles in later stages of cardiac LR determination, such as in the propagation of asymmetric signals from the LPM to the heart primordium.

In this paper, we conduct detailed studies of Wnt/β-catenin signaling in LR determination and reveal its unique functions in regulating cardiac laterality. We identify Wnt3 and Wnt8 as two canonical Wnts that are expressed in the KV region and influence

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LR patterning by regulating both KV and midline development. Importantly, we reveal a function of Wnt/β-catenin signaling in a later step of cardiac LR patterning that modulates coordinated expression between Southpaw (Spaw), the Nodal homolog in zebrafish, and Lefty2. Finally, we identify Gata4 as a downstream mediator that is regulated by Wnts and controls the competence of the heart field to respond to asymmetric cues.

MATERIALS AND METHODS

Zebrafish strains

Wild-type (TL), transgenic *gata4::gfp*, and heterozygous *apc*^{mcr} fish lines were used for this work. Genotyping of the homozygous *apc*$^{mcr/mcr}$ mutation was performed according to protocols from H. Clevers' Laboratory (Utrecht University, Utrecht, The Netherlands).

Morpholino injections

Antisense morpholino oligonucleotides against *apc* (Nadauld et al., 2004), *wnt3* (Shimizu et al., 2005), *wnt8* (Lekven et al., 2001), *gata4* (Holtzinger and Evans, 2005), *spaw* (Long et al., 2003) and *gata4* (e2) (targeting the *e2* splicing donor site of *gata4*: 5′-TTGCAATTTTCTCACCAGTCGTCTC-3′) were obtained from Gene Tools. We optimized the dosage of morpholinos, so the majority of embryos exhibited reported phenotypes without severe embryonic malformation; these doses were 2 ng for the *apc* MO, 5 ng for the *wnt3* MO, 5 ng for the *wnt8* MO, 8 ng for the *gata4* (ATG) MO, 2 ng for the *gata4* (e2) MO, and 4 ng for the *spaw* MO, unless specified otherwise in the Results.

To analyze the effect of *gata4* (e2) MO injection on mRNA splicing, the following primers were used. Primer pair e1 (5′-CTTCGACAG-CTCCGTACTGC-3′) and e3 (5′-TGGAGCTTCATGTAGAGTCC-3′) amplify fragments of normal splicing or exon2 skipping; primer pair e2 (5′-AACCGGCCGCTGGTCAAACC-3′) and i2 (5′-CAAGTGCACT-CAATCAATCC-3′) amplify a product of intron retention. To quantify *gata4* mRNA levels, real-time PCR analysis was carried out as described previously (Lin et al., 2007) using forward (5′-TTTTGATGATC-TGGGCGAGGGC-3′) and reverse (5′-TCTCCTTCTGCATTGCGTC-TCC-3′) primers.

Cloning and RNA injections

Full-length zebrafish *gata4* and *dickkopf 1* (*dkk1*) cDNA were amplified by an expand high-fidelity PCR system (Roche), using 24 hpf cDNA as a template. The resulting cDNA fragments were cloned into *pCS2+* plasmid.

Capped mRNAs were synthesized from *pCS2+* plasmids containing the desired genes using the SP6 mMESSAGE mMACHINE kit (Ambion), and 10-20 pg of *wnt3* RNA, 10-20 pg of *wnt8* RNA, 25 pg of *dkk1* RNA or 1-15 ng of *gata4* RNA was injected into one- or two-cell staged embryos.

Lithium chloride treatment

Lithium chloride (LiCl) treatment was carried out as previously described (Carl et al., 2007; Kim et al., 2002).

In situ hybridization

Two-color fluorescent hybridization was performed as previously described (Clay and Ramakrishnan, 2005). Briefly, digoxigenin (Roche)-labeled *gata4* riboprobe and fluorescein (Roche)-labeled *nkx2.5* riboprobe were co-hybridized with embryos. The first and second fluorescent signals were developed sequentially using Tyramide Signal Amplification Kit (Molecular Probes) and imaged using an LSM 510 confocal microscope (Zeiss, Germany). Single-color whole-mount in situ hybridization was conducted as previously described (Xu et al., 2002).

Antibody staining

KV cilia were visualized by antibody staining using anti-acetylated tubulin antibody (Sigma) as described (Essner et al., 2005). After staining, the tail region was removed, flat-mounted, and photographed by using an Axioplan II Zeiss microscope equipped with Apotome. Cilia length was measured using AxioVision software.

RESULTS

APC is involved in the establishment of LR asymmetry

To examine functions of Wnt/β-catenin signaling in laterality, we first analyzed zebrafish *apc*$^{mcr/mcr}$ mutant embryos, which harbor a nonsense mutation in *apc*, an essential intracellular negative regulator of Wnt/β-catenin signaling. In the heart, both cardiac jogging and cardiac looping was disrupted; this was indicated by a failure of the atrium to move towards the left side at 28 hours post-fertilization (hpf; Fig. 1B,E) in 93% (*n*=57) of homozygous mutant embryos, and 95% (*n*=115) of the ventricles failed to loop to the right of the atrium at 52 hpf (Fig. 1E,H). In contrast to the heart, the visceral organs were positioned normally despite their developmental defects. For example, the pancreas was located correctly on the right side of mutant embryos, as revealed by in situ hybridization of *trypsin* (100%, *n*=59; Fig. 1D,E); the liver was also localized normally, as revealed by *prox1* staining (98%, *n*=48; images not shown). In the brain, asymmetry seemed also disrupted, as suggested by the expression of *lov,* a left-side dominant gene in habenula (Gamse et al., 2005), which became bilaterally symmetric in day 4 mutants (100%, *n*=46; images not shown). In summary, the above data suggest that activation of the Wnt/β-catenin pathway affects the LR asymmetry of heart and brain, but not of visceral organs.

Activation and repression of Wnt/β-catenin signaling results in distinct laterality defects

In zebrafish, the expression of w*nt3* and *wnt8*, but not other canonical Wnts, has been reported in the tail bud region (Shimizu et al., 2005). By performing co-staining of Wnts with *charon*, a novel Cer/Dan family member of the Nodal antagonists that is specifically expressed in a subset of cells in the KV (Hashimoto et al., 2004), we detected *wnt3* and *wnt8* in both the tail bud (see Fig. S1A-D in the supplementary material) and the vicinity of KV (see Fig. S1C-F in the supplementary material), suggesting that they have functions in KV development and LR asymmetry. Therefore, we carried out both gain-of-function and loss-of-function studies for these two genes. Gain-of-function experiments were performed by injection of *wnt3* or *wnt8* RNA into single-cell staged embryos. To exclude the possibility that asymmetry defects are due to perturbed anterior dorsal development as described previously (Danos and Yost, 1995), we optimized the injection dosage so that brain and eye structures still existed. Injection of 10-20 pg of RNA resulted in a lack of jogging (*wnt3* RNA: 39%, *n*=165; *wnt8* RNA: 36%, *n*=148; see Fig. S2B,D in the supplementary material) and a lack of looping (*wnt3* RNA: 51%, *n*=114; *wnt8* RNA: 67%, *n*=83; Fig. 1H,L) phenotypes in the heart. By contrast, the laterality of visceral organs, such as liver, gut and pancreas, was mostly unaffected, with only a small percentage of embryos showing opposite or bilateral expression of liver markers (*wnt3* RNA: 7% opposite, 3% bilateral, *n*=114; *wnt8* RNA: 7% opposite, 7% bilateral, *n*=83; Fig. 1I,J,L). Therefore, the activation of Wnt/β-catenin signaling by overexpression of either *wnt3* or *wnt8* is able to recapitulate the lack of cardiac laterality phenotypes observed in *apc* mutants.

To investigate the endogenous roles of *wnt3* and *wnt8*, loss-of-function studies were carried out by injection of previously characterized anti-*wnt3* and anti-*wnt8* morpholinos (Lekven et al., 2001; Shimizu et al., 2005). In *wnt3* morphants, both cardiac jogging (51% L-jog, 23% No-jog, 26% R-jog, *n*=133; see Fig. S2 in the supplementary material) and looping (52% D-loop, 24% No-loop, 24% L-loop, *n*=72; Fig. 1F-H,L) were randomized. Laterality in visceral organs was also randomized (57% normal, 19% midline,

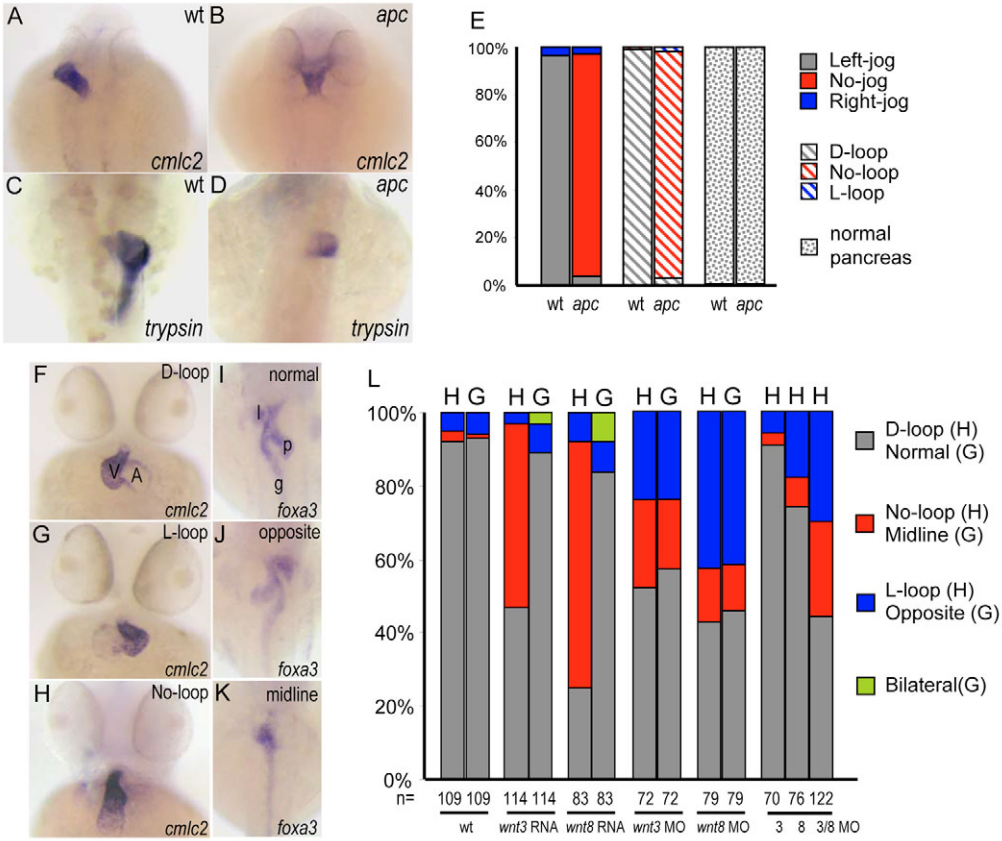


Fig. 1. Wnt/β-catenin signaling regulates LR asymmetry. (**A-E**) APC is required for left-right patterning of zebrafish heart but not visceral organs. Wild-type sibling (A,C) and *apc* mutant (B,D) embryos were stained at 28 hpf (A,B) for *cmlc2* and at 52 hpf (C,D) for *trypsin*. (E) Quantification of cardiac jogging and looping, and pancreas positioning. Shown are dorsal views with anterior to the top. (**F-L**) Activation and repression of Wnt/β-catenin signaling differentially affect cardiac and visceral organ laterality. (F-H) Representative images of cardiac looping as revealed by *cmlc2* staining of 52 hpf embryos. Ventral views with anterior to the top. (I-K) Representative images of the positioning of visceral organs as revealed by *foxa3* staining of 52 hpf embryos. Dorsal views with anterior to the top. l, liver; g, gut; p, pancreas. (L) Quantification of cardiac and visceral organ laterality. H, heart; G, gut and other visceral organs; *n*, total numbers of embryos scored.

24% opposite, *n*=72; Fig. 1I-L). Similarly, *wnt8* morphants displayed randomized hearts (50% L-jog, 23% No-jog, 27% R-jog, *n*=107; 42% D-loop, 15% No-loop, 43% L-loop, *n*=79) and visceral organ laterality (45% normal, 13% midline, 42% opposite, *n*=79; Fig. 1F-L; see also Fig. S2 in the supplementary material). We next performed double knockdown experiments. Although the injection of lower doses of *wnt3* or *wnt8* morpholinos (1 ng) resulted in either no or only a slight effect on heart asymmetry, the co-injection of *wnt3* and *wnt8* morpholinos led to greatly enhanced LR defects (Fig. 1L, last group), indicating that *wnt3* and *wnt8* coordinately regulate LR asymmetry. We conclude that a reduction of Wnt/β-catenin signaling randomizes laterality in both heart and visceral organs.

Both *wnt3* and *wnt8* are required for KV development and midline expression of *lefty1*

To gain mechanistic insights into distinct laterality phenotypes in embryos with activated and repressed Wnt signaling, we examined the expression of *charon,* ciliogenesis, and the perinodal expression of *spaw*, three key indicators of KV LR patterning function (Bisgrove et al., 2005; Essner et al., 2005; Gourronc et al., 2007; Hashimoto et al., 2004; Raya et al., 2003). The expression of *charon* was not affected by injection of either *wnt3* or *wnt8* RNA (Fig. 2A-C). Similarly, none of the progenies derived from intercrossing $apc^{mcr/+}$ fish exhibited abnormal expression of *charon* (data not shown). The injection of either *wnt3* or *wnt8* RNA resulted in larger KV and an increased cilia number (*wnt3* RNA, 79±14; *wnt8* RNA, 77±13), when compared with wild-type control embryos (56±9; Fig. 2G-I,N), although cilia length was not affected (*wnt3* RNA, 5.9±0.4 μm; *wnt8* RNA, 6.2±0.5 μm; versus 6.3±0.4 μm in wild-type control embryos; Fig. 2G-I,M). Conversely, *charon* expression was suppressed in *wnt3* morphants (30/48) and *wnt8* morphants (24/54), and was nearly absent in *wnt3/wnt8* double morphants (17/19; Fig. 2D-F). Cilia length was significantly reduced in *wnt3* morphants (5.3±0.5 μm), *wnt8* morphants (4.5±0.6 μm), and *wnt3/wnt8* double morphants (4.0±0.4 μm), although cilia numbers remained normal (*wnt3* morphants, 54±10; *wnt8* morphants, 58±11; *wnt3/wnt8* morphants, 54±8; Fig. 2J-N). Additionally, the perinodal expression of *spaw* in 12- to 14-somite staged embryos was unaltered in *apc* mutants and in embryos injected with *wnt3* or *wnt8* RNA (data not shown), but was downregulated in 79% (*n*=29) of *wnt3* morphants and 94% (*n*=50) of *wnt3/wnt8* double morphants (images not shown). In summary, the activation or repression of Wnt/β-catenin signaling differently affects KV development, which might be partially responsible for the distinct laterality phenotypes observed.

We next examined midline development, because it might affect LR patterning (Bisgrove et al., 1999; Bisgrove et al., 2000). The formation of midline tissue was not affected in embryos with either activated or repressed Wnt signaling, as indicated by the normal expression of *ntl* in the notochord and *axial* in the floor plate (see Fig. S3A-P in the supplementary material). We also examined the midline expression of *lefty1*, because it was thought to serve as a molecular barrier (Meno et al., 1998) and is absent in Wnt3a-deficient mice (Nakaya et al., 2005). Interestingly, the expression of *lefty1* in the notochord showed no discernible difference from wild type in *apc* morphants or in either *wnt3* or *wnt8* RNA-injected embryos, but expression was abolished in embryos injected with *wnt3* (8/14) or *wnt8* (16/18) morpholinos (see Fig. S3Q-T in the supplementary material; data not shown). Thus, Wnt/β-catenin signaling is dispensable for the formation of the midline tissues but is required for the midline expression of *lefty1*. The different effects on midline development upon activation or repression of Wnt signaling could also account for their distinct laterality phenotypes.

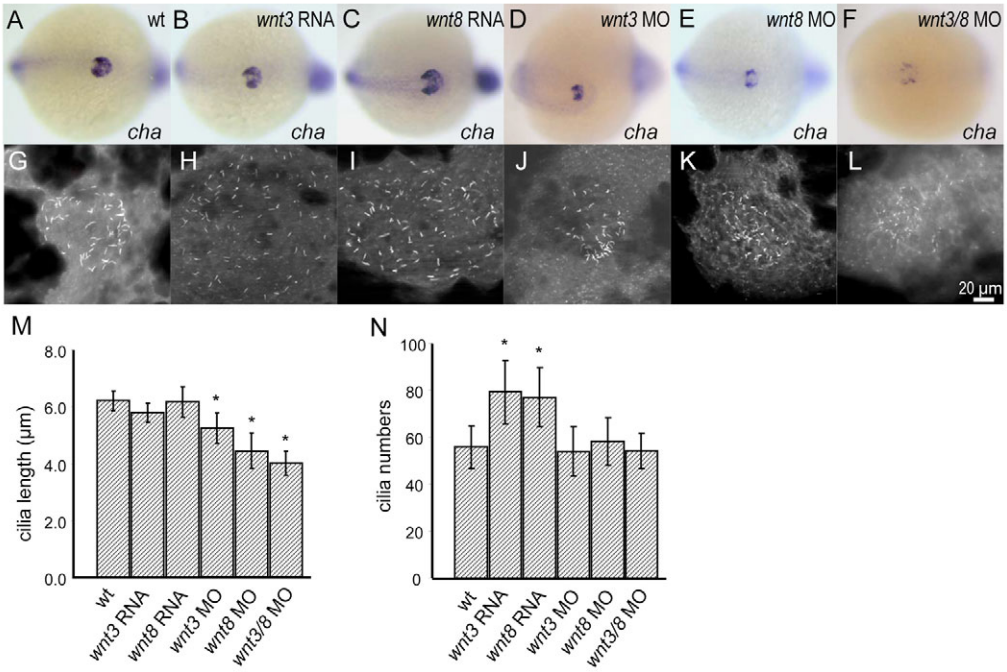


Fig. 2. Wnt/β-catenin signaling is essential for the LR patterning function of KV. (**A-F**) *charon* expression was suppressed in *wnt3* (D), *wnt8* (E), and *wnt3/wnt8* double (F) morphants, but not in embryos overexpressing *wnt3* (B) or *wnt8* (C) RNA, as compared with control embryos (A). Shown are dorsal views of tail bud regions at the 12-somite stage. (**G-N**) Cilia development in KV was regulated by Wnt signaling, as shown by anti-acetylated tubulin antibody staining of 10-somite staged embryos. Compared with controls (G; *n*=15), shorter KV cilia were observed in *wnt3* morphants (J; *n*=15), *wnt8* morphants (K; *n*=12), and *wnt3/wnt8* double morphants (L; *n*=15), but not in embryos injected with *wnt3* RNA (H; *n*=12) or *wnt8* RNA (I; *n*=12), as summarized in M. Cilia numbers were increased in embryos overexpressing *wnt3* (H) or *wnt8* (I) RNA but remained the same in *wnt3* (J), *wnt8* (K) and *wnt3/wnt8* double (L) morphants, as summarized in N. Data shown are means±s.d. **P*<0.001, Student's *t*-test.

Activation of Wnt/β-catenin signaling disrupts *lefty2* expression in the heart field without affecting *spaw* expression in the LPM

To further understand the molecular mechanisms of Wnt signaling in regulating laterality, we examined the expression of left-side-specific genes, including *spaw* in the LPM, *lefty2* in the heart primordium and *pitx2* in the posterior LPM. The injection of *wnt3* or *wnt8* RNA did not have significant effects on *spaw* expression, which remained left-sided in 87% of *wnt3*-overexpressing embryos (*n*=115) and 94% of *wnt8*-overexpressing embryos (*n*=82; Fig. 3A,I,Q). By contrast, *lefty2* expression was absent in 45% of embryos injected with *wnt3* RNA (*n*=157) and 36% of embryos injected with *wnt8* RNA (*n*=120), without significant right-sided or bilateral occurrence (Fig. 3H,L,Q). Consistent with this observation, left-sided *spaw* expression was not perturbed in 99% of the progeny (*n*=148) derived from intercrossing $apc^{mcr/+}$ heterozygous fish, although *lefty2* expression was absent in 24% of the progeny (*n*=148). The identity of the embryos without *lefty2* expression was later confirmed by genotyping to be homozygous $apc^{mcr/mcr}$ mutants. To obtain further evidence to demonstrate that activation of the Wnt pathway disrupts *lefty2* expression in the heart field without affecting *spaw* expression in the LPM, we incubated embryos with LiCl, a GSK3β inhibitor, to temporally control the activation of Wnt/β-catenin signaling. Indeed, a lack of *lefty2* expression could be seen when LiCl was administrated as early as mid-gastrulation (56%, *n*=68) and as late as 8- to 10-somite stages (8 somites: 37%, *n*=85; 10 somites: 29%, *n*=77), after KV had formed, whereas *spaw* expression remained normal (see Table S2 in the supplementary material). To quantify the correlation between *spaw* expression and *lefty2* expression, we calculated the co-efficiency index (CEI) after co-staining of *spaw* and *lefty2* on 22- to 24-somite staged embryos (Fig. 3I-L). The CEI was defined as being the number of embryos showing same-sided expression of *spaw* and *lefty2* per total numbers of embryos scored. As summarized in Fig. 6I, the CEI was reduced from 0.92 in wild-type embryos to 0.64 in embryos injected with *wnt3* RNA and 0.67 in embryos injected with *wnt8* RNA, respectively. Lack of side-specific gene expression seemed to be specific to the heart field and not the gut primordium, as indicated by normal *pitx2* expression in the posterior LPM in embryos injected with *wnt3* RNA (89% left, 4% absent, 3% right, 4% bilateral, *n*=87) or *wnt8* RNA (89% left, 7% right, 4% bilateral, *n*=47; Fig. 3M-Q), and in embryos derived from intercrossing $apc^{mcr/+}$ heterozygous fish (99% left, *n*=71). In summary, the normal expression of *spaw* in the LPM and *pitx2* in the posterior LPM suggests that activation of the Wnt/β-catenin pathway did not affect the early steps of laterality, despite its function in increasing KV size and KV cilia numbers. Instead, the lack of a cardiac asymmetry phenotype seems to result from the disturbance of a specific later step, when the asymmetric signal propagates from Spaw to Lefty2.

To investigate the roles of repressed Wnt signaling on asymmetric signal propagation, we examined side-specific gene expression in *wnt3* and *wnt8* morphants. In *wnt3* morphants, the expression of both *spaw* and *lefty2* was largely absent (76% for *spaw*; 79% for *lefty2*; *n*=33) at the 19- to 21-somite stage, and then became randomized

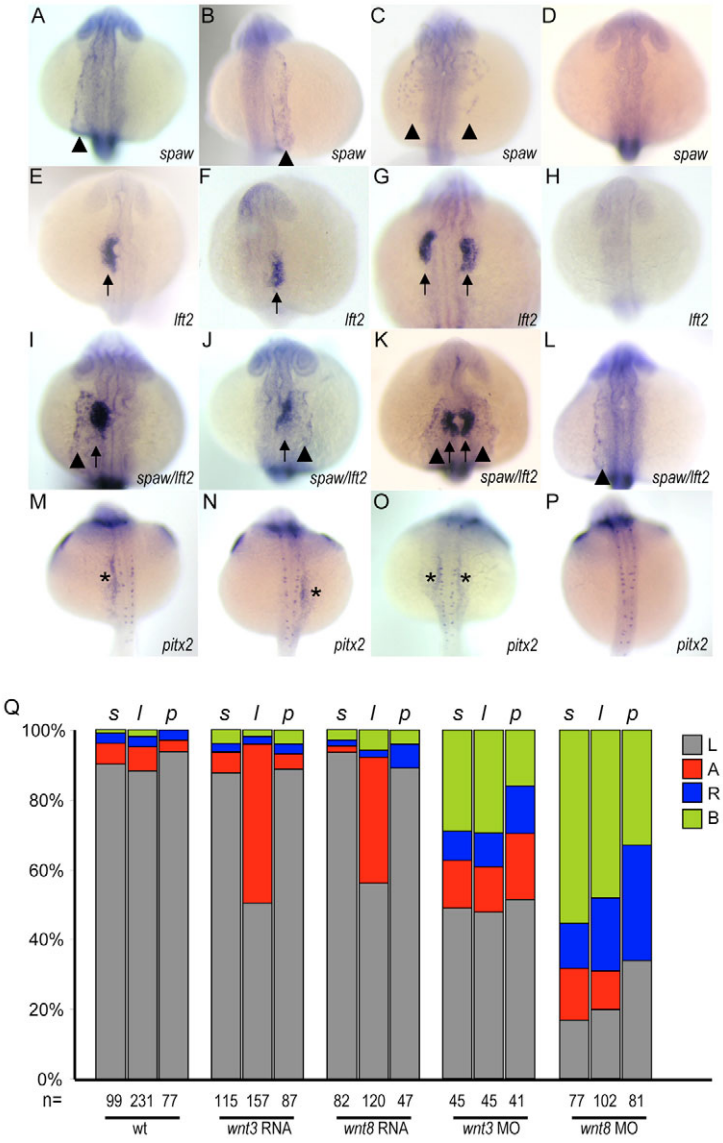


Fig. 3. Activation of Wnt signaling ablates *lefty2* expression in the heart without affecting the left-sided expression of *spaw* and *pitx2*. (**A-P**) Representative images of *spaw* expression in LPM (arrowheads in A-C,I-L), *lefty2* expression in the cardiac field (arrows in E-G,I-K), and *pitx2* expression in the posterior LPM (asterisks in M-O). Shown are dorsal views, with anterior to the top of 22- to 24-somite staged embryos. A *tropomyosin* probe was included in in situ hybridization to ensure embryos were at the proper stage, with the exception of *pitx2* staining. (**Q**) Quantification of the percentages of asymmetric expression of *spaw*, *lefty2* and *pitx2*. *s*, *spaw*; *l*, *lefty2*; *p*, *pitx2*. L, left side; A, absence; R, right side; B, bilateral; *n*, total numbers of embryos scored.

(*spaw*: 49% left, 14% absent, 8% right, 29% bilateral, *n*=45; *lefty2*: 47% left, 13% absent, 10% right, 30% bilateral, *n*=45) at the 22- to 24-somite stage (Fig. 3A-L,Q). Delayed *spaw* expression in the LPM might be due to a reduced level of perinodal *spaw*, as was seen and suggested in the Wnt3a-deficient mouse study (Nakaya et al., 2005). The expression of *pitx2* was also randomized (51% left, 19% absent, 14% right, 16% bilateral, *n*=41) at the 22- to 24-somite stage (Fig. 3M-Q). Similar results were obtained in *wnt8* morphants (*spaw*: 17% left, 15% absent, 13% right, 55% bilateral, *n*=77; *lefty2*: 20% left, 11% absent, 21% right, 48% bilateral, *n*=102; *pitx2*: 34% left, 33% right, 33% bilateral, *n*=81; Fig. 3Q). Although bilateral expression of *spaw* was prevalent in *wnt3* and *wnt8* morphants, which probably resulted from a lack of *lefty1* expression in the notochord, right-sided expression did exist, which could be due to defective KV development (Fig. 2). Thus, the perturbed LR patterning seen in embryos with reduced Wnt/β-catenin signaling is due to defects in both KV development and *lefty1* expression in the embryonic midline. In contrast to embryos with activated Wnt signaling, the CEI values were normal in *wnt3* morphants (0.92) and in *wnt8* morphants (0.94; Fig. 6I), indicating that the correlated expression between *spaw* and *lefty2* was not disrupted.

gata4 expression in the heart field is regulated by Wnt/β-catenin signaling

We reasoned that disrupted propagation of asymmetric signals from the LPM to the heart field upon activation of the Wnt pathway might result from a disturbed capacity of the heart field to respond to *spaw* in the LPM, and that, if so, we might be able to detect gene expression changes within the heart field. To test this hypothesis, we examined the expression of a panel of cardiogenic factors, including *nkx2.5*, *gata4*, *gata5*, *gata6* and *tbx5*, in 12-somite staged embryos, prior to the onset of *spaw* and *lefty2* expression in the LPM (Fig. 4; data not shown). To facilitate our analysis, we employed a previously characterized anti-*apc* morpholino instead of using *apc* mutants (Nadauld et al., 2004). We found that the expression of *gata4* was dramatically reduced in the caudal region of the anterior LPM (ALPM) upon injection of either the *apc* morpholino or *wnt3* RNA (Fig. 4A-C). By contrast, the expression of *gata4* was extended caudally and laterally in embryos injected with *dkk1* RNA (Fig. 4E), and was unchanged in *wnt3* morphants (Fig. 4D), probably because of a redundant function of other canonical Wnts. We did observe an anterior shift of the ALPM in embryos injected with the *apc* morpholino (Fig. 4B,G,L,Q) and to

a lesser extent in embryos injected with *wnt3* RNA (Fig. 4C,H,M,R). However, we consider it unlikely that the response of *gata4* to Wnt signaling was a consequence of the known function of the Wnt/β-catenin pathway in anteroposterior polarity patterning because the other cardiogenic factors, such as *nkx2.5*, *gata5*, *gata6* and *tbx5* (Fig. 4F-O; data not shown), and an early cardiac-specific sarcomere gene, *ttna* (Fig. 4P-T) (Seeley et al., 2007), showed normal expression. In fact, injection of the *apc* morpholino resulted in a partial exclusion of *gata4* from the *nkx2.5* expression domain and its lateral region, as demonstrated by two-color fluorescent in situ hybridization (Fig. 4W1,X1,Y1).

To examine whether *gata4* expression is regulated by Wnt signaling at the transcriptional level, we analyzed a *gata4::gfp* transgenic fish line that harbors a *gfp* reporter driven by a 14.8-kb *gata4* enhancer. Despite ectopic notochord expression, this transgenic fish line was able to recapitulate endogenous *gata4* expression in the ALPM and heart (Heicklen-Klein and Evans, 2004). As expected, *gfp* expression in the ALPM was diminished from the caudal region upon injection of the *apc* morpholino or *wnt3* RNA (Fig. 4V; data not shown), which was similar to the whole-mount *gata4* in situ hybridization results. The discovery that *gata4* expression is regulated by the Wnt pathway at the transcriptional level, together with the previous demonstration of a no-looping heart in Gata4-deficient animals (Holtzinger and Evans, 2005; Watt et al., 2004), led us to speculate that Gata4 mediates Wnt-controlled cardiac laterality.

Gata4 mediates the cardiac laterality defect in embryos with activated Wnt signaling

To investigate the function of Gata4 in the establishment of cardiac asymmetry, a loss-of-function study was performed using a previously characterized anti-*gata4* (ATG) morpholino (Holtzinger and Evans, 2005) and a newly synthesized morpholino that targets a splicing donor site (e2). Here, we present results using the anti-*gata4* (e2) morpholino because it can be used in rescuing experiments by co-injection with *gata4* RNA, and because the two morpholinos gave rise to identical phenotypes. Injection of the anti-*gata4* (e2) morpholino resulted in both exon skipping and intron retention (Fig. 5A), and consequently reduced the wild-type *gata4* transcript by 97.2%, as revealed by real-time PCR analysis (Fig. 5A). Similar to those with activated Wnt signaling, the hearts in embryos injected with

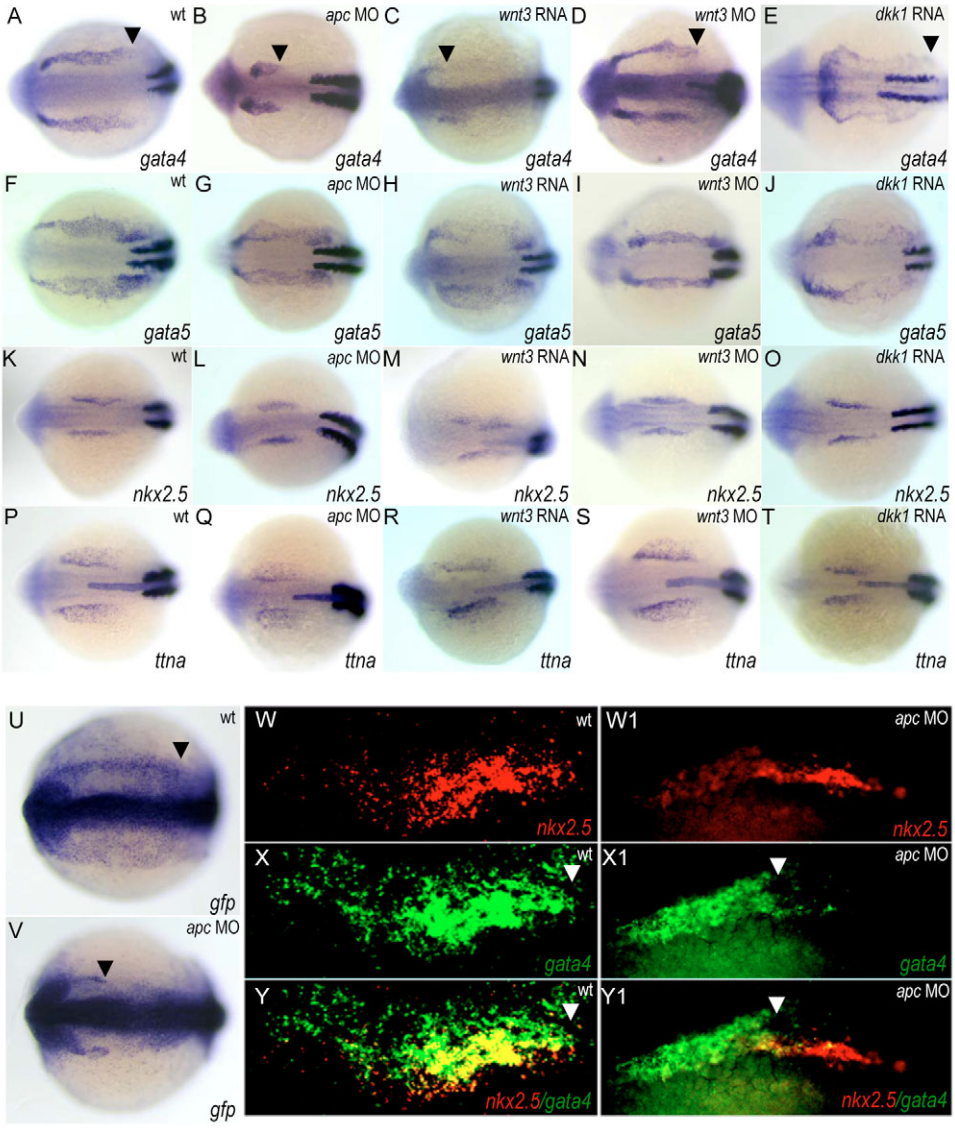


Fig. 4. Extent of gata4 expression responds to Wnt/β-catenin signaling. (**A-T**) Wnt/β-catenin signaling regulates *gata4* expression. (A-E) *gata4* expression was restricted to the rostral ALPM in *apc* morphants (B) and in embryos injected with *wnt3* RNA (C), was unaltered in *wnt3* morphants (D), and was expanded caudally in *dkk1*-overexpressing embryos (E), compared with controls (A). (F-J) *gata5* expression, (K-O) *nkx2.5* expression, and (P-T) *ttna* expression were not affected. The embryos were co-stained with a *myod* probe to ensure the proper developmental stage. (**U**,**V**) Wnt signaling modulates *gata4* expression at the transcriptional level. The expression of *gfp* in *gata4::gfp* transgenic fish was revealed by in situ hybridization using a *gfp* riboprobe. (**W-Y1**) Two-color fluorescent in situ hybridization showed that *gata4* expression was partially depleted from the *nkx2.5* expression domain and its lateral region in *apc* morphants (W1-Y1), compared with in controls (W-Y). Shown are dorsal views of 12-somite staged embryos with anterior to the left; only the right LPM is shown in W-Y1. Arrowheads indicate the posterior end of *gata4* expression.

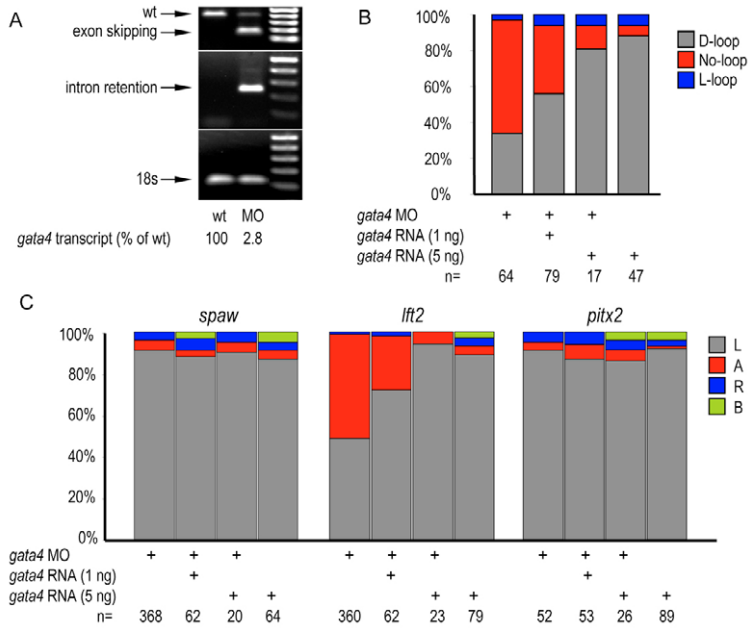
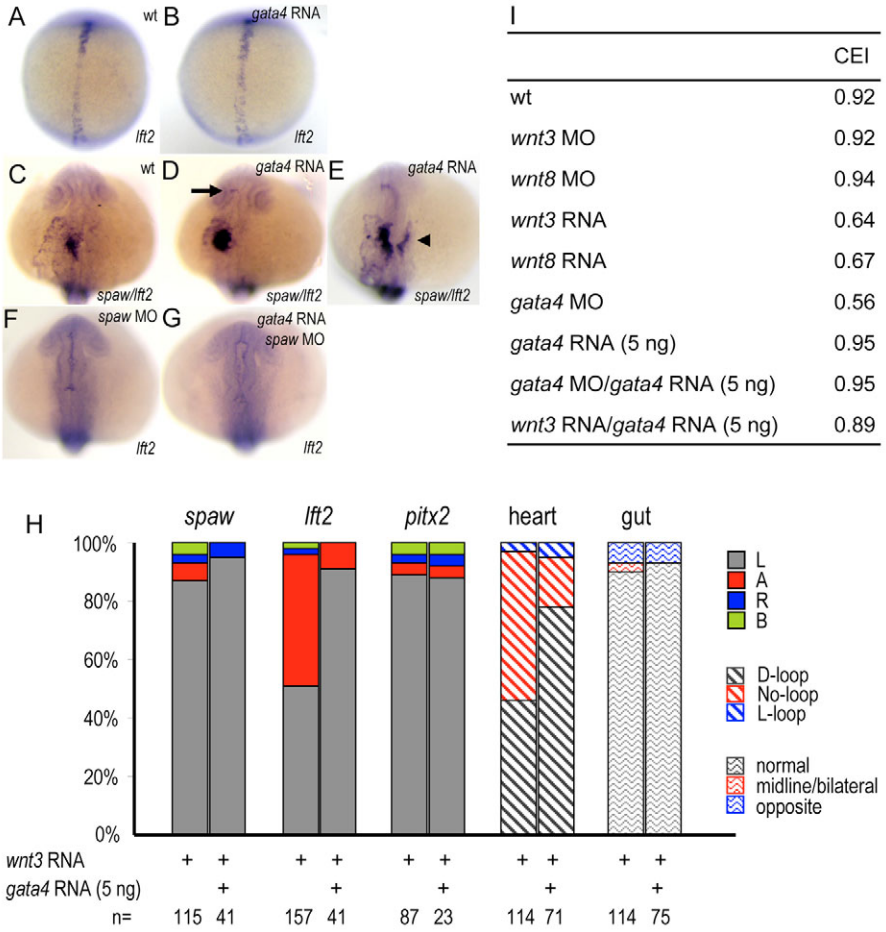


Fig. 5. Gata4 is required for cardiac LR patterning. (**A**) The *gata4* (e2) morpholino efficiently disrupted mRNA splicing. RT-PCR on mRNA from morpholino-injected embryos amplified a 188-bp product (exon 2 skipping) and a 268-bp product (intron 2 retention); RT-PCR from control embryos only amplified a 454 bp product (normal splicing). Normally spliced *gata4* mRNA in these morphants was 2.8% of the wild-type level as revealed by real-time PCR analysis. (**B**,**C**) Reducing Gata4 resulted in laterality defects, which can be rescued by co-injection with *gata4* RNA in a dose-dependent manner. (B) Quantification of cardiac looping. (C) Quantification of asymmetric expression of *spaw*, *lefty2* and *pitx2*.

anti-*gata4* (e2) morpholino exhibited the no-jogging (33%, *n*=165) and, subsequently, the no-looping (63%, *n*=64) phenotype (Fig. 5B; see Table S1 in the supplementary material). By contrast, the gut laterality remained normal (see Table S1 in the supplementary material), despite suppressed visceral organ development as revealed by *transferrin* and *foxa3* staining (Holtzinger and Evans, 2005) (data not shown). This cardiac-specific laterality defect was unlikely to be due to disrupted KV function, because *charon* expression (see Fig. S4B in the supplementary material), ciliogenesis in the KV (see Fig. S4E,J,K), and *spaw* (91% left, 5% absent, 4% right, *n*=368) and *pitx2* (91% left, 4% absent, 5% right, *n*=52) expression (Fig. 3A,M; Fig. 5C; see also Table S1 in the supplementary material) were all normal. It was also not due to abnormal midline formation, as indicated by the presence of both physical markers, such as *ntl* and *axial* (see Fig. S4H in the supplementary material; data not shown), and molecular markers, i.e. *lefty1* (data not shown). However, *gata4* morphants exhibited an absence of *lefty2* expression in the heart field (48% left, 51% absent, 1% right, *n*=360; Fig. 3H,L; Fig. 5C; see also Table S1 in the supplementary material). This cardiac laterality defect can be rescued by *gata4* RNA in a dosage-dependent manner. Co-injection of 1 ng of *gata4* RNA with the *gata4* morpholino reduced the percentage of embryos with no looping heart from 63% (*n*=64) to 38% (*n*=79), and restored the percentage of embryos with *lefty2* expression from 48% (*n*=360) to 72% (*n*=62; Fig. 5B,C; see Table S1 in the supplementary material). The co-injection of 5 ng of *gata4* RNA further reduced the percentage of embryos with no looping heart to 13% (*n*=17), and restored the percentage of embryos with *lefty2* expression to 94% (*n*=23; Fig. 5B,C; see Table S1 in the supplementary material). Importantly, the correlated expression between *spaw* and *lefty2* was rescued, as indicated by the increase of CEI from 0.56 in *gata4* morphants to 0.79 in embryos co-injected with 1 ng of *gata4* RNA and to 0.95 in embryos co-injected with 5 ng of *gata4* RNA (Fig. 6I). At the same time, injection of up to 5 ng of *gata4* RNA alone did not elicit any defects in gene expression (Fig. 5B,C; see Fig. S4 and Table S1 in the supplementary material). In summary, these data confirm a specific function of Gata4 in cardiac laterality and demonstrate that a reduction of Gata4 recapitulates the laterality phenotypes observed in embryos with activated Wnt signaling.

Having demonstrated that the reduction of Gata4 disrupted *lefty2* expression without disturbing *spaw* expression, we then explored whether overexpression of *gata4* was sufficient to induce *lefty2* expression. We found that an injection of 5 ng of *gata4* RNA into wild-type embryos enhanced *lefty2* expression in the left diencephalon (25/39; Fig. 6D, arrow) and that an injection of 15 ng of *gata4* RNA further induced *lefty2* expression in the right cardiac field (7/23; Fig. 6E, arrowhead). The enhanced or ectopic *lefty2* expression was likely to be Nodal dependent. First, it could not be observed at stages from 50% epiboly to 3 somites (Fig. 6A,B; data not shown), prior to the onset of *spaw* expression (Long et al., 2003). Second, it was only detected in areas in which *lefty2* expression can be initiated by *spaw*. To test this hypothesis, we examined whether *gata4* can induce *lefty2* expression in *spaw* morphants. A previously described anti-*spaw* morpholino (Long et al., 2003) was injected alone or together with *gata4* RNA into wild-type embryos. In *spaw* morphants, *lefty2* expression was absent (99/99; Fig. 6F) and could not be restored by the injection of up to 15 ng of *gata4* RNA (60/60; Fig. 6G). These data indicate that *gata4* induces *lefty2* expression in a Spaw-dependent manner.

Finally, to examine the hypothesis that Gata4 is the cardiogenic factor that mediates activated Wnt/β-catenin pathway in the heart, we investigated whether *gata4* RNA can rescue the cardiac laterality defects seen in embryos with activated Wnt signaling. Indeed, the co-injection of *gata4* and *wnt3* RNA reduced the percentage of embryos with no-looping heart to 17% (*n*=71), compared with 51% (*n*=114) in embryos injected with *wnt3* RNA alone (Fig. 6H; see also Table S1 in the supplementary material). The percentage of embryos that lacked *lefty2* expression was also reduced to 9% (*n*=41), compared with 45% (*n*=157) in embryos overexpressing *wnt3* alone, although the expression of *spaw* and *pitx2*, as well as visceral organ laterality, were not affected by *gata4* overexpression (Fig. 6H; see Table S1). The correlated expression between *spaw* and *lefty2* was rescued, as indicated by the increase of CEI from 0.64 in embryos injected with *wnt3* RNA alone to 0.89 in embryos co-injected with



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	CEI
wt	0.92
wnt3 MO	0.92
wnt8 MO	0.94
wnt3 RNA	0.64
wnt8 RNA	0.67
gata4 MO	0.56
gata4 RNA (5 ng)	0.95
gata4 MO/*gata4* RNA (5 ng)	0.95
wnt3 RNA/*gata4* RNA (5 ng)	0.89

Fig. 6. Gata4 mediates the function of Wnt signaling in regulating cardiac laterality. (**A-G**) Overexpression of *gata4* induces *lefty2* expression in a *spaw*-dependent manner. (A,B) Injection of *gata4* RNA had no effect on *lefty2* expression in floorplate precursors (B), compared with controls (A). Shown are dorsal views of 3-somite staged embryos with anterior to the top. (C-E) Injection of *gata4* RNA induced *lefty2* expression in diencephalon (D, arrow) and in the right heart field (E, arrowhead). (F,G) Injection of *spaw* morpholino resulted in a lack of *lefty2* expression (F), which cannot be restored by *gata4* RNA (G). Shown are dorsal views of 22-somite staged embryos with anterior to the top. (**H**) Overexpression of *gata4* restored *lefty2* expression and cardiac looping defects in embryos injected with *wnt3* RNA. Shown is the quantification of side-specific gene expression, as well as heart and gut looping. L, left side; A, absence; R, right side; B, bilateral; *n*, total numbers of embryos scored. (**I**) Summary of CEI. Correlated expression between *spaw* and *lefty2* was calculated by the co-efficiency index (CEI). Between 17 and 360 embryos were scored for each experimental group.

gata4 RNA (Fig. 6I). These genetic results strongly suggest that Gata4 mediates Wnt/β-catenin signaling in regulating cardiac LR patterning.

DISCUSSION

In this paper, we investigated the role of Wnt/β-catenin signaling in the establishment of cardiac asymmetry and revealed its distinct functions in KV development and asymmetric signal transmission to the heart field (Fig. 7). As detailed below, our data prompt a hypothesis that, in contrast to the instructive role of Nodal (Spaw), Wnt/β-catenin signaling plays a permissive role in the ALPM to regulate the competence of the heart field to respond to Spaw.

Functions of Wnt/β-catenin signaling in KV development and cardiac asymmetry

Our data revealed that a reduction of either Wnt3 or Wnt8 disrupted the development and LR patterning function of KV, as indicated by suppressed *charon* expression, shortened cilia length, reduced perinodal *spaw* expression, and later randomized *spaw* expression in the LPM. Wnt3a-deficient mouse also exhibited defective LR patterning function of the node, probably because of the reduction of *polycystin* (*PC1*) expression and the restriction of *Nodal* expression to the posterior edge of the ventral node. However, cilia structure in the node remained normal (Nakaya et al., 2005). The different effect on KV development between the two species may provide an explanation for why *wnt3* morphants exhibited certain right-sided expression of *spaw*, *lefty2* and *pitx2* besides bilateral expression, whereas the Wnt3a-knockout mouse displayed only bilateral expression of these genes (Nakaya et al., 2005).

Conversely, overexpression of *wnt3* or *wnt8* led to morphological changes in KV, including an increased size and increased cilia number. Consistent with this observation, the ectopic activation of Wnt signaling in mice carrying a mutated version of *apc* resulted in the formation of expanded node (Ishikawa et al., 2003). However, this observation is at odds with two previous reports, where KV was either normal (Bajoghli et al., 2007) or reduced/absent (Schneider et al., 2008) upon activation of Wnt signaling. Surprisingly, despite morphological changes in KV, the activation of Wnt signaling in either *apc* mutants or embryos overexpressing *wnt3* or *wnt8* did not affect the LR patterning function of KV, as indicated by normal *charon* and *spaw* expression around KV and in the LPM. Consistently, normal left-sided *spaw* expression in the LPM was observed in *mbl* mutants (Carl et al., 2007).

We showed that the activation of Wnt/β-catenin signaling led to a loss of cardiac asymmetry. Similar no-looping hearts have also been reported in embryos with enhanced Wnt signaling, such as *gsk3β* morphants and *mbl* mutants (Carl et al., 2007; Lee et al., 2007). Together, these studies challenge previous reports that the activation of Wnt signaling results in a reversal of heart looping and a predominantly bilateral expression of Nodal (Bajoghli et al., 2007; Danos and Yost, 1995; Rodriguez-Esteban et al., 2001). Multiple reasons could account for the discrepancy, including species difference, dosage difference, or ligand-specific effects. It is also

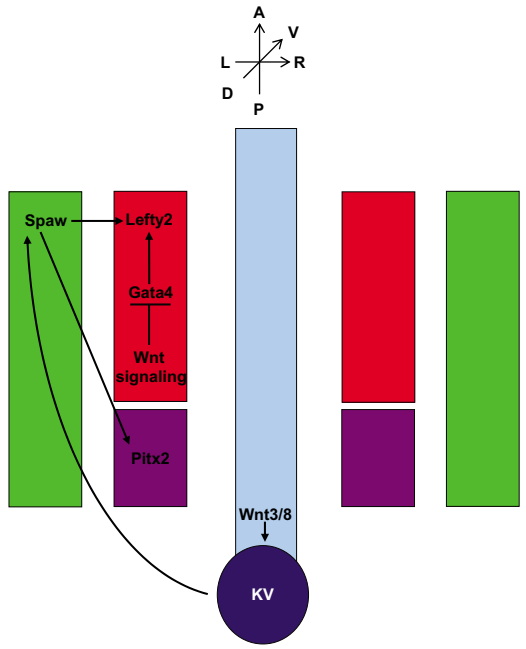

Fig. 7. Model for the LR patterning function of Wnt/β-catenin signaling. Wnt/β-catenin signaling affects laterality at two steps. First, Wnt3 and Wnt8 are expressed in the KV region and regulate the development of KV, which sequentially affects laterality in both heart and visceral organs. Second, Wnt/β-catenin signaling negatively regulates Gata4 expression in the cardiac field, which subsequently modulates Lefty2 expression in the cardiac primodium. In contrast to the inductive role of Spaw, Wnt/β-catenin signaling imposes a permissive function on the cardiac field, allowing it to respond to asymmetric cues such as Spaw. This latter function of Wnt signaling affects only heart and not the visceral organs. Blue, midline; Red, heart primordium; Green, LPM; Purple, gut primordium.

possible that other pathways in addition to Wnt were activated, as many of the components of the Wnt pathway, for example β-catenin and GSK3β, are used in other signaling pathways (Hayward et al., 2008). At least in some cases, the reversal of heart looping was secondary to dorsal-anterior defects, a well-known characteristic of early Wnt activation (Danos and Yost, 1995). We did observe a reversal of heart looping in embryos with severe dorsal-anterior defects caused by the injection of higher amounts of *wnt3* or *wnt8* RNA. Therefore, we carefully controlled the RNA doses to avoid such severe malformation in the embryos.

Given the controversies in the roles of Wnt/β-catenin signaling in KV development and cardiac asymmetry, further investigations and in depth analyses are needed. Mutant embryos will certainly provide invaluable insights into this matter. Additionally, the genetic manipulation of different components in the Wnt pathway should be conducted and compared in parallel, and the amount of RNA or morpholino used should be carefully considered to avoid secondary defects.

Target organ-specific functions of Wnt/β-catenin signaling in LR determination

At the molecular level, the activation of Wnt/β-catenin signaling resulted in the absence of *lefty2* expression in the context of normal *spaw* and *pitx2* expression. This suggested that asymmetric signal propagation from *spaw* in the LPM to *lefty2* in the heart field, but not to *pitx2* in the posterior LPM, was disrupted. The differentially affected expression of *lefty2* and *pitx2* might eventually lead to a no-looping heart and normally positioned visceral organs. Our discovery demonstrated that Wnt/β-catenin signaling plays distinct LR patterning functions in different target organs. Consistent with this notion, laterality in the brain but not visceral organs is affected by the overactivation of Wnt/Axin1/β-catenin signaling during late gastrulation (Carl et al., 2007). As in brain, Wnt activity needs to be kept at a relative low level in the heart to ensure the proper establishment of cardiac asymmetry. However, the functional mechanism of Wnt activity in the heart appears to be different from that in the brain (Carl et al., 2007).

Adding to the complexity of Wnt functions, Wnt/β-catenin signaling plays an additional uncharacterized role in determining organ laterality, as suggested by the observation that the activation of Wnt/β-catenin signaling during a narrow window of mid-somite stages disrupted the expression of Nodal pathway genes concordantly in the epithalamia and the LPM (Bajoghli et al., 2007; Carl et al., 2007). A similar regulation of Nodal expression, independent of node and midline function, was reported in the Man1-deficient mouse, in which TGFβ signaling was disrupted (Ishimura et al., 2008). Taken together, Wnt/β-catenin signaling executes multiple and distinct functions in regulating target organ laterality, which warrant further investigation.

Gata4 in the heart field mediates Wnt-regulated cardiac laterality

Gata4 belongs to a zinc-finger transcription factor family, which is important in cardiogenesis and cardiac hypertrophy (Bisping et al., 2006; Oka et al., 2006; Watt et al., 2004; Zeisberg et al., 2005). Our data revealed a novel function of Gata4 in the asymmetric signal propagation from Spaw to Lefty2, and placed it downstream of the Wnt/β-catenin pathway. We consider it less likely that Gata4 affects cardiac laterality by functioning downstream of Spaw, as the onset of *gata4* expression in the heart is around the 5-somite stage on both sides of the LPM, which is much earlier than the left-sided expression of *spaw* in the LPM. Instead, Spaw and Gata4 are more likely to function as two independent signals that converge at Lefty2. Therefore, we propose that Wnt-Gata4 is a permissive signaling pathway that regulates cardiac laterality by modulating the competence of the heart field to respond to asymmetric cues in the LPM. This combinatory mechanism was supported by the observation that injection of *gata4* RNA induced ectopic *lefty2* expression in wild-type embryos, but not in *spaw* morphants, in a context-dependent manner, preferably in regions that are already under Spaw control. However, Gata4 might not be the immediate early response gene for Wnt signaling, as activation of Wnt signaling can influence cardiac asymmetry prior to the onset of Gata4 expression, as indicated by the observation that LiCl administration as early as mid-gastrulation was able to suppress *lefty2* expression.

Why does the activation of Wnt/β-catenin signaling regulate heart but not visceral organ asymmetry? One possibility is that the difference is due to the timing of the onset of *gata4* expression in different organ primordia. *gata4* expression occurs prior to the initiation of *lefty2* in the heart primordium, but at around the time of *pitx2* initiation in the intestinal epithelium, at 14 to 19 somites (Shin et al., 2007). Although we cannot rule out that other factors might play a role, our discovery provides novel leads for further study of the functions of both Gata4 and Wnt in heart morphogenesis, which promises to reveal the molecular mechanisms responsible for human congenital heart diseases, especially those with heterotaxy.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/207/DC1

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