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Supplemental Data

Wnt/Axin1/β-Catenin Signaling Regulates Asymmetric Nodal Activation, Elaboration, and Concordance of CNS Asymmetries

Matthias Carl, Isaac H. Bianco, Baubak Bajoghli, Narges Aghaallaei, Thomas Czerny, and Stephen W. Wilson

Supplemental results

Early activation of Wnt/β-catenin signalling disrupts asymmetric Nodal pathway gene expression

To address if modulation of Wnt signalling can affect asymmetry we activated (exogenous *wnt8b*, *activated-β-catenin* or *tcf3a* morpholinos) the pathway early in development. A well-described consequence of such manipulations is to disturb AP patterning of the CNS with Wnt signalling posteriorising the rostral brain (Wilson and Houart, 2004; Yamaguchi, 2001). To minimise this effect, we used low amounts of reagents so that we could assess asymmetric structures in embryos in which regionalisation of the brain is relatively normal.

Overexpression of *wnt8b* resulted in concordant bilateral Nodal pathway gene expression in both the LPM and the epithalamus (Figure S1A-B and data not shown). At later stages, embryos showed heterotaxic laterality defects in viscera, heart and brain (Figure S1C-F).

Both the expression of *activated-β–catenin* and interference with *tcf3a* function induced AP patterning phenotypes similar to those obtained by *wnt8b* overexpression but far fewer embryos exhibited laterality defects (Figure S1A''-F''). Some *tcf3a* morphants showed reversed expression of the habenula marker *lov,* but most other markers were relatively unaffected (Figure S1D'').

Several Wnt ligands and Wnt pathway genes are expressed in the epithalamus

At least five Wnt ligands are expressed in the epithalamus by the 10 to 14 somite stage (Figure S3) and *wnt16* is expressed in this region soon afterwards (Thisse and Thisse, 2004). Using *flh* expression to define the presumptive pineal complex (Concha et al., 2003; Masai et al., 1997), we find that *wnt1*, *wnt10b* (Lekven et al., 2003; Thisse and Thisse, 2005) and *wnt3a* are expressed caudal and lateral to the presumptive pineal complex (Figure S3A-D), a *wnt7b* homologue (Experimental Procedures) and *wnt8b* are expressed ventral to this domain (Figure S3E-H) and *wnt2* is expressed ubiquitously (Thisse and Thisse, 2004). In addition, several genes that function downstream in the Wnt/ß-catenin pathway are expressed in and/or around the epithalamus at these

stages (Figure S3 and e.g. (Dorsky et al., 2003; Dorsky et al., 1999; Kim et al., 2000; Nasevicius et al., 2000; Veien et al., 2005; Young et al., 2002).

Wnt genes continue to be expressed in the epithalamus after brain asymmetries become morphologically evident with *wnt1* and *wnt7b* expression detectable close to the epiphysis and *wnt3a* and more strongly *wnt8b* expression in the habenulae (Figure S3K-R). At the level of transcription, we did not observe any obvious asymmetries in expression of Wnt pathway components in the brain.

dkk, *axin1* and *axin2* are also expressed in and around the epithalamus at somite stages (Figure S3I,J,S,U) and both *axin* genes continue to be expressed in the epithalamus throughout the first few days of development (Figure S3T-V).

Figure legends for supplementary data:

Figure S1. Manipulations that disrupt early Wnt pathway activity result in loss of asymmetry of Nodal pathway gene expression in the LPM and brain and heterotaxia phenotypes

(A-F) The panels show wild-type expression of genes as indicated on the left. (A'-F') To illustrate the effect of manipulation of Wnt signalling, the panels show the expression of the same genes in *wnt8b* injected embryos. (A,A') Frontal views of the epithalamus and (B,B') dorsal views of 24s stage embryos. Red dotted line indicates the midline, black arrows point at *pitx2* expression in the epithalamus (et) and white arrows at *pitx2* expression in the lateral plate mesoderm (LPM).

(C,C',D,D') Dorsal views of the epithalamus at (C,C') 2 days and (D,D') 4 days of development. (E,E') Dorsal and (F,F') frontal views of 2 days old embryos. The yellow dotted line outlines (E,E') the embryo and (F,F') the heart.

(A''-F'') The graphs illustrate the percentage of embryos with wild-type (WT) left, reversed (rev) right, bilateral (bil), medial (med) or not visible (nv) gene expression upon activation of the Wnt signalling pathway.

lhab, left habenula; pp, parapineal; rhab, right habenula.

Figure S2. Left and right habenular axon terminals are segregated along the DV axis of the target IPN nucleus

Dorsal view of the interpeduncular nucleus (IPN) in a 7 days old medaka embryo. DiI/DiD labelling of axonal projections from the habenulae shows the dorso-ventral segregation within the IPN similar to zebrafish (compare with e. g. Figure 4I). dIPN, dorsal and vIPN, ventral IPN.

Figure S3. Expression of various Wnt pathway genes in and around the epithalamus.

(A-K,M,O,Q,S-V) Lateral views and (L,N,P,R,T',V') dorsal views focused on the diencephalic epithalamus (et; except S,U). All developmental stages are indicated on the left of each horizontal row of panels and the marker genes used on the top of each vertical row. (A-J) *floating head* (*flh*) expression in the epithalamus (in red) has been used as a landmark. (I,J) *dickkopf* (*dkk*) is expressed in the epithalamic area at mid-somitogenesis stages. Its expression fades shortly later (data not shown). (S,T,T') In zebrafish and Xenopus *axin1* is initially expressed ubiquitously up to tailbud stages but becomes subsequently confined to the fore- and midbrain region (Hedgepeth et al., 1999; Van de Water et al., 2001). (T') Similar to Xenopus we find increased *axin1* expression in the habenula from three days of development onward, but more pronounced on the left side (red dotted circle). (U,V,V') *axin2* is expressed in the dorsal neural tube and CNS including the epithalamus

also at later stages. For further details see supplementary results. ey, eye; hab, habenula (green circle); tc, telencephalon; tec, tectum.

Figure S4: Mosaic overactivation of Wnt signalling can induce *lft1*

(A,B) Lateral views of the presumptive tail region of tailbud stage embryos, which have been injected with GFP:HS:lef-VP16 DNA (see also Bajoghli et al., 2007), heat shock treated at late gastrulation and 2 hrs later analysed for *lft1* and GFP expression. Ectopic *lft1* expression can be seen in the vicinity of GFP expressing cells.

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Table S1: Analysis of asymmetry in *masterblind* (*axin1)* mutant embryos

marker	wt	reversed	bilateral	not visible	n
lft1-brain	32 (100)	0 (0)	68 (0)	0 (0)	82 (62)
pitx2-brain	29 (99)	1 (0)	70 (1)	0 (0)	105 (94)
cyc-brain	23 (93)	3 (1)	68 (0)	6 (6)	31 (88)
otx5-pp	38 (100)	5 (0)	0 (0)*	57 (0)	111 (124)
gfi-pp	39 (nd)	13 (nd)	36 (nd)*	12 (nd)	83 (nd)
cxcr4b-hab	85 (86)	13 (12)**	2 (2)**	0 (0)	53 (50)
zic2a-hab	100 (100)	0 (0)**	0 (0)**	0 (0)	15 (20)
lov-hab	5 (96)	3 (2)	92 (2)	0 (0)	134 (54)
tag1-hab	10 (88)	19 (8)	71 (4)	0 (0)	87 (135)
dex-hab	2 (90)	12 (3)	86 (7)	0 (0)	64 (31)
α-acet.tubulin-hab	2 (95)	6 (0)	88 (5)	4 (0)	51 (79)
DiI/DiD-IPN	0 (100)	0 (0)	96 (0)***	4 (0)	25 (2)
pitx2-LPM	100 (nd)	0 (nd)	0 (nd)	0 (nd)	82 (nd)
spw-LPM	94 (96)	2 (0)	2 (2)	2 (3)	54 (118)
cmlc2-heart	84 (95)	0 (0)	16 (5)*	0 (0)	62 (64)
fkd2-liver	98 (100)	0 (0)	2 (0)*	0 (0)	62 (64)
insulin-pancreas	100 (100)	0 (0)	0 (0)*	0 (0)	62 (64)

Analysis of *spw* was performed at 16s, *lft1*, *pitx2* and *cyc* was performed at 24s, *cxcr4b*, *otx5*, *gfi*, *zic2a*, *cmlc2*, *fkd2* and *insulin* at 2 days, *tag1* at 3 days and *lov*, *dex*, α-acetylated tubulin and DiI/DiD at 4 days; analysis of *masterblind* siblings is shown in brackets. *, medial expression instead of bilateral; **, left instead of reversed; right instead of bilateral; ***, ventral instead of bilateral acet., acetylated; hab, habenula; IPN, interpeduncular nucleus; nd, not determined; n, number of embryos analysed; pp, parapineal; numbers in brackets show values for *masterblind* siblings; all scores shown in column 2-5 show percentages

Table S2. Embryos treated with LiCl show stage dependent disruption of lateralised Nodal pathway gene expression

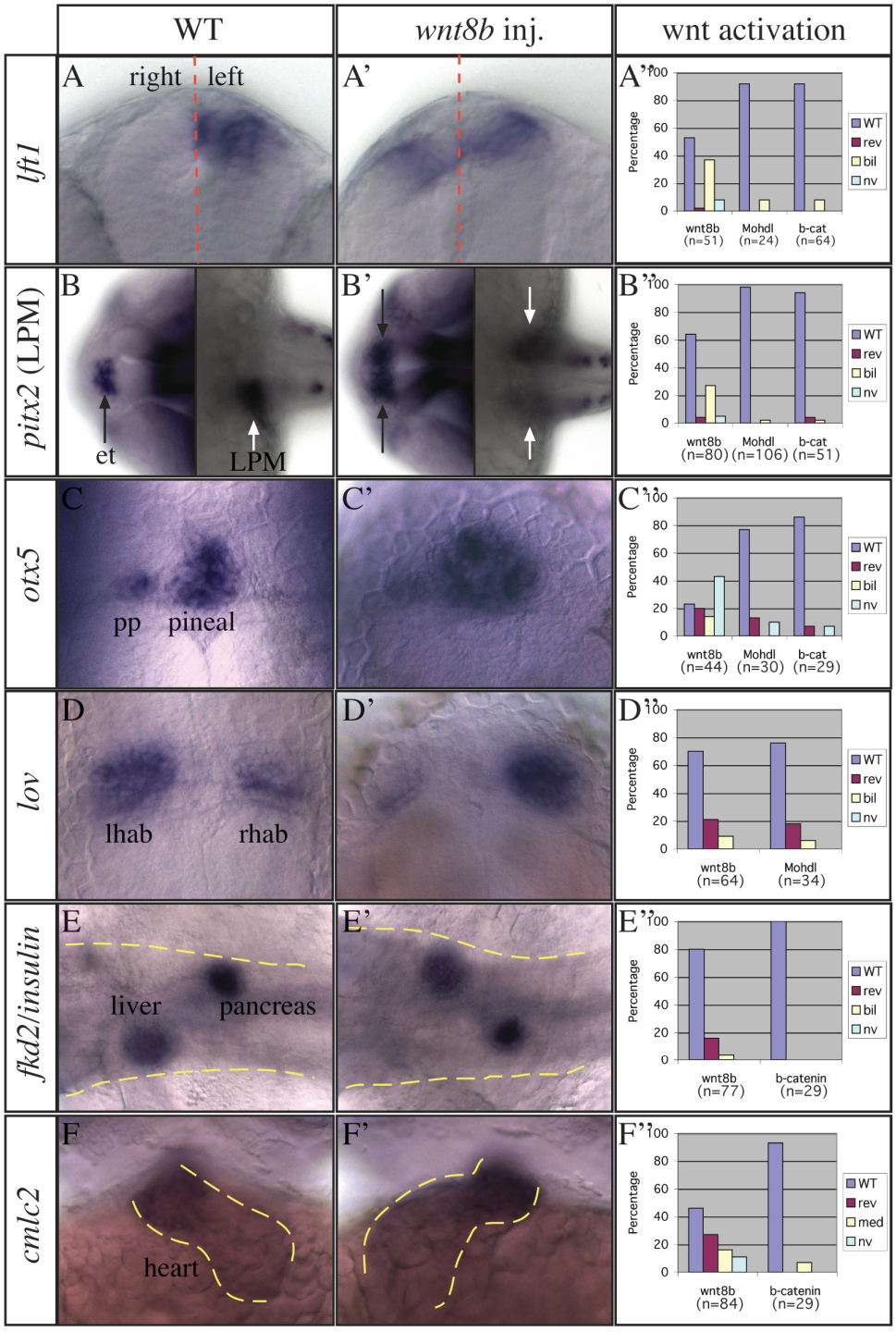
stage/marker	wt (left)	reversed (right)	bilateral	not visible	n
LiCl-80%					
lft1	67	2	31	0	67
pitx2(in LPM)	80 (100)	2 (0)	18 (0)	0 (0)	137(52)
otx5	30	7	4*	59	111
lov	89	8	3	0	172
LiCl-90%					
lft1	91	0	3	6	34
pitx2(in LPM)	88 (91)	5 (8)	7 (1)	0 (0)	86 (89)
otx5	78	5	2*	15	81
lov	94	6	0	0	32
LiCl-tb					
lft1	95	3	2	0	58
pitx2(in LPM)	90 (100)	5 (0)	5 (0)	0 (0)	38 (40)
otx5	94	4	0	2	49
lov	94	6	0	0	33
LiCl-12s					
lft1	69	16	15	0	67
pitx2(in LPM)	64 (nd)	13 (nd)	23 (nd)	0 (nd)	31 (nd)
otx5	66	19	0*	15	90
lov	82	13	5	0	93
LiCl-14s					
lft1	51	20	29	0	99
pitx2 (in LPM)	50 (67)	12 (20)	38 (13)	0 (0)	72 (64)
otx5	68	19	1*	12	131
lov	63	32	5	0	85
LiCl-18s					
lft1	94	1	5	0	108
pitx2(in LPM)	96 (100)	1 (0)	3 (0)	0 (0)	67 (49)
otx5	92	3	0*	8	149
lov	78	17	5	0	218
LiCl-22s					
lft1	84	3	13	0	46
pitx2(in LPM)	89 (100)	0 (0)	11 (0)	0 (0)	37 (27)
otx5	92	5	0*	3	60
lov	85	14	1	0	94
LiCl-26hpf					
otx5	99	0	1*	0	78
lov (30hpf)	95 (96)	5 (0)	0 (4)	0 (0)	38 (44)
LiCl-36hpf					
otx5	100	0	0*	0	28
lov	100	0	0	0	40

Embryos were treated with LiCl for 20 min (15 min when earlier than tailbud stage) and fixed at 24s (*lft1*, *pitx2*), 2,5 days (*otx5*) and 4 days (*lov*). *, medial expression instead of bilateral; n, number of embryos scored; nd, not determined
all scores in column 2-5 show percentages

TableS3: Inhibition of GSK-3β with LiCl at 14s results in loss of nodal gene asymmetry

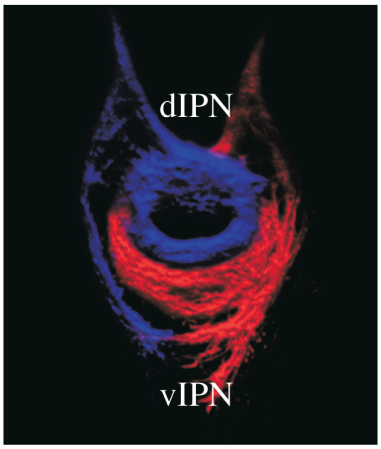
marker	wt	reversed	bilateral	not visible	n
lft1-brain	51	20	29	0	99
pitx2-brain	50	12	38	0	72
otx5-pp	77	22	0*	1	115
lov-habenula	63	32	5**	0	85
pitx2-LPM	67	20	13	0	64
cmlc2-heart	70	18	12*	0	89
fkd2-liver	62	25	13*	0	89
insulin-pancreas	62	21	17*	0	89

Wild-type embryos were LiCl treated at 14s stage for 20 min
All embryos were analysed at 24s (*lft1*, *pitx2*), 2,5 days (*otx5*, *cmlc2*, *fkd2*, *insulin*) and 4 days (*lov*).
*, medial expression instead of bilateral;**, bilateral right instead of bilateral; n, number of embryos analysed; pp, parapineal; all scores in column 2-5 show percentages

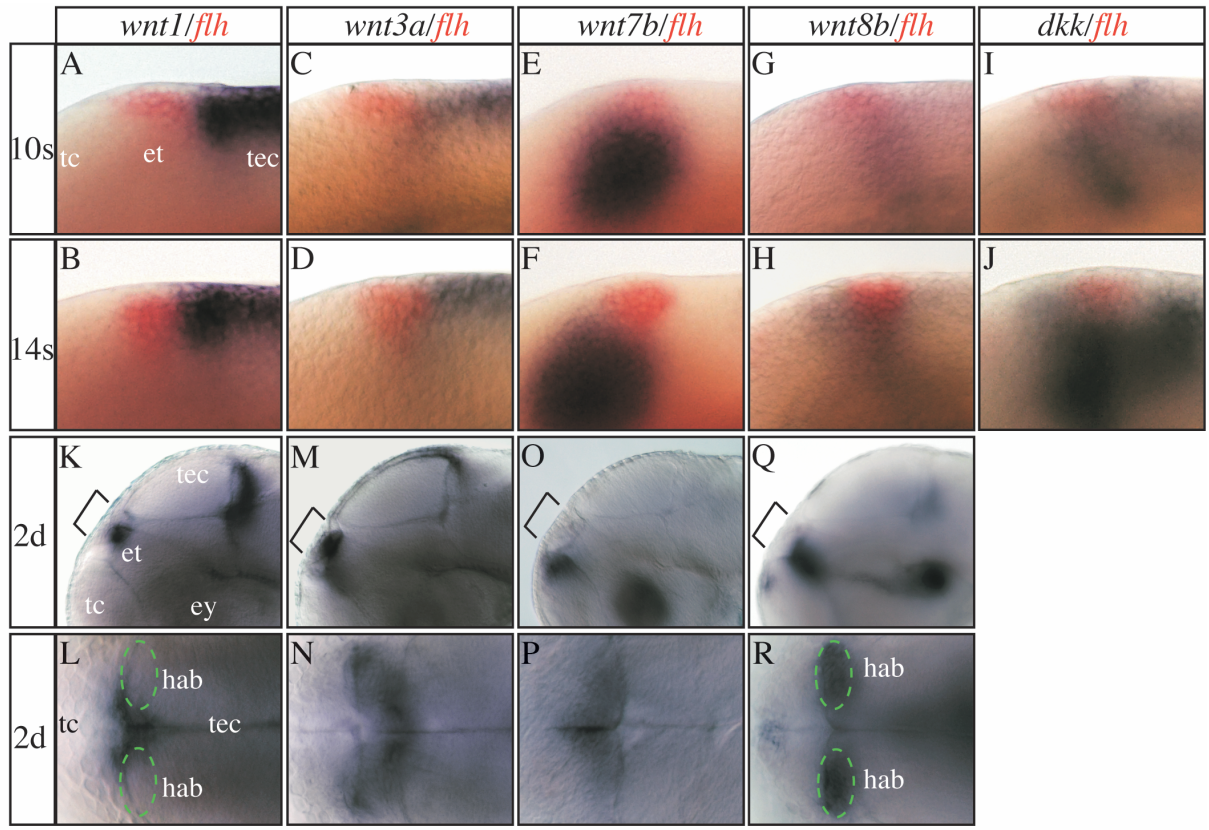


Carl et al., Supplementary Fig. 1

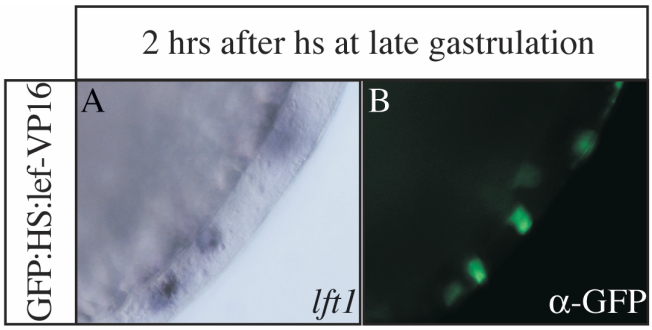
DiI/DiD labelling in medaka



Carl et al., Supplementary Fig. 2



Carl et al., Supplementary Fig. 3



Carl et al., Supplementary Fig. 4