Data Supplement

Supplementary Figures

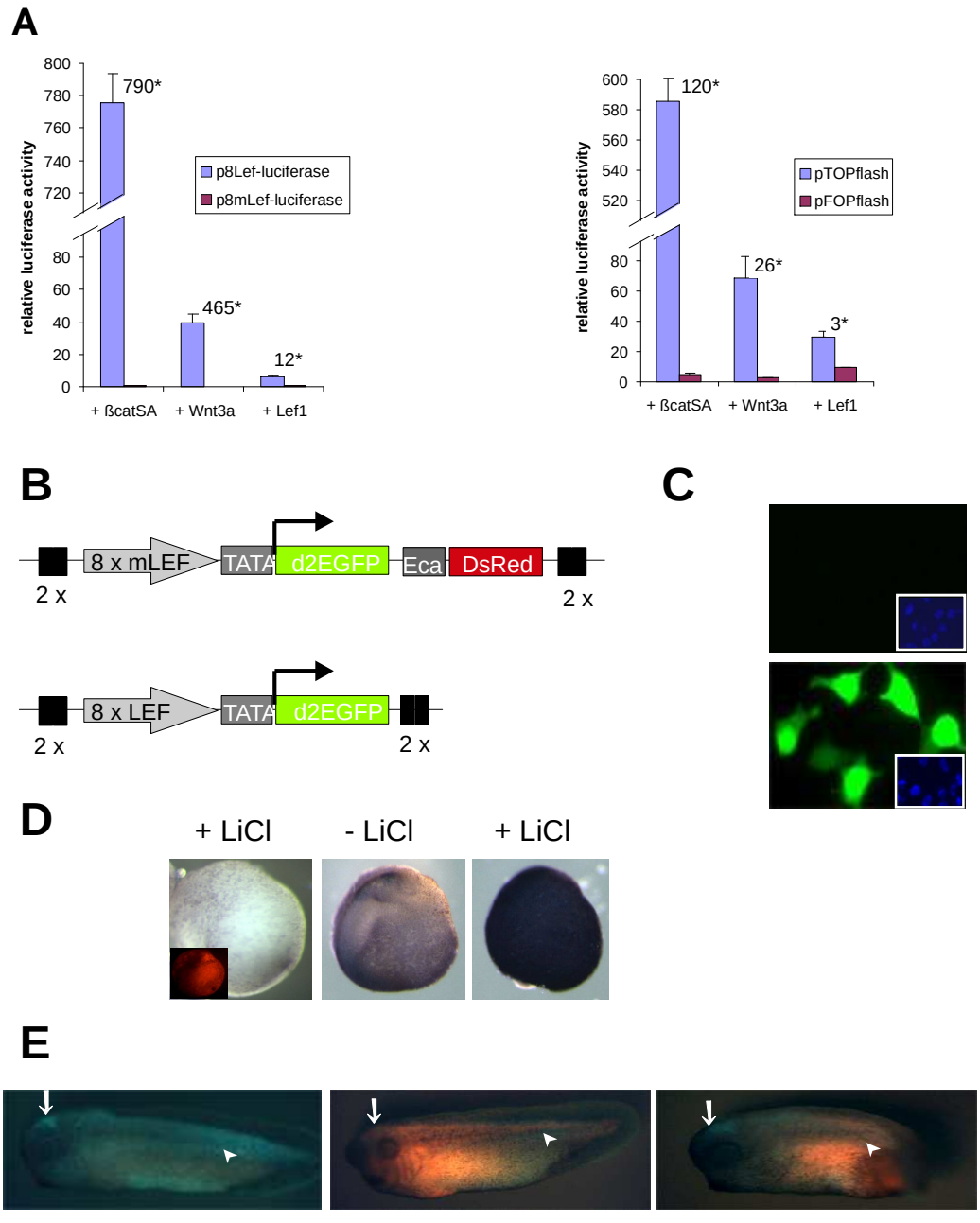

Figure S1. Validating the activity and specificity of Wnt-reporter constructs in cell culture and in transgenic *Xenopus* embryos.

(**A**) The induction of the p8Lef-luciferase plasmid in response to Wnt stimulation is comparable to that of the pTOPflash plasmid. Importantly, the mutant p8mLef-luciferase plasmid exhibited much lower basal activities than pFOPflash. The ratio between the activities of the p8Lef-luciferase and p8mLef-luciferase plasmids is indicated by the number near the standard deviation bars. Significance of differences between means was analyzed by a Student's t-test (* $p < 0.05$). (**B**) Schematic overview of pbin8mLef-GFP-EcDsRed (control construct) and pbin8Lef-GFP (Wnt/β-catenin reporter construct). Eca: minimal promoter of the human E-cadherin gene, which drives expression of the DsRed gene in the epidermal tissues. (**C**) Expression of GFP in 293T cells when the control (top panel) and the reporter (bottom panel) plasmids were contransfected with a stabilized form of β-catenin. (**D**) Transgenic control (left panel) and reporter embryos (middle and right panel) were treated with 0.3M LiCl for 10 min and processed by WISH using a digoxigenin-labeled d2EGFP probe. Stage 21 Wnt-reporter transgenic *X. tropicalis* embryos express GFP transcripts ubiquitously in response to LiCl stimulation (right panel). No signal is detected in the transgenic control embryo (inset left panel: DsRed expression used to identify the transgenic embryos). (**E**) Transgenic Wnt-reporter embryos were injected at the 4-cell stage with 100 pg RNA of a Wnt-inhibiting construct (pEnR-LEFΔN-GR) either in the dorsal (middle panel) or the ventral side (right panel). Rhodamine dextran sulfate was coinjected as a tracer. Embryos were treated with 10 μM Dex at stage 28 and visualized at stage 32. Treated embryos responded to Wnt inhibition and could be directly analyzed *in vivo*. Arrows: GFP signal at the midbrain-hindbrain boundary; arrowheads: position of GFP expression in the posterior somites.

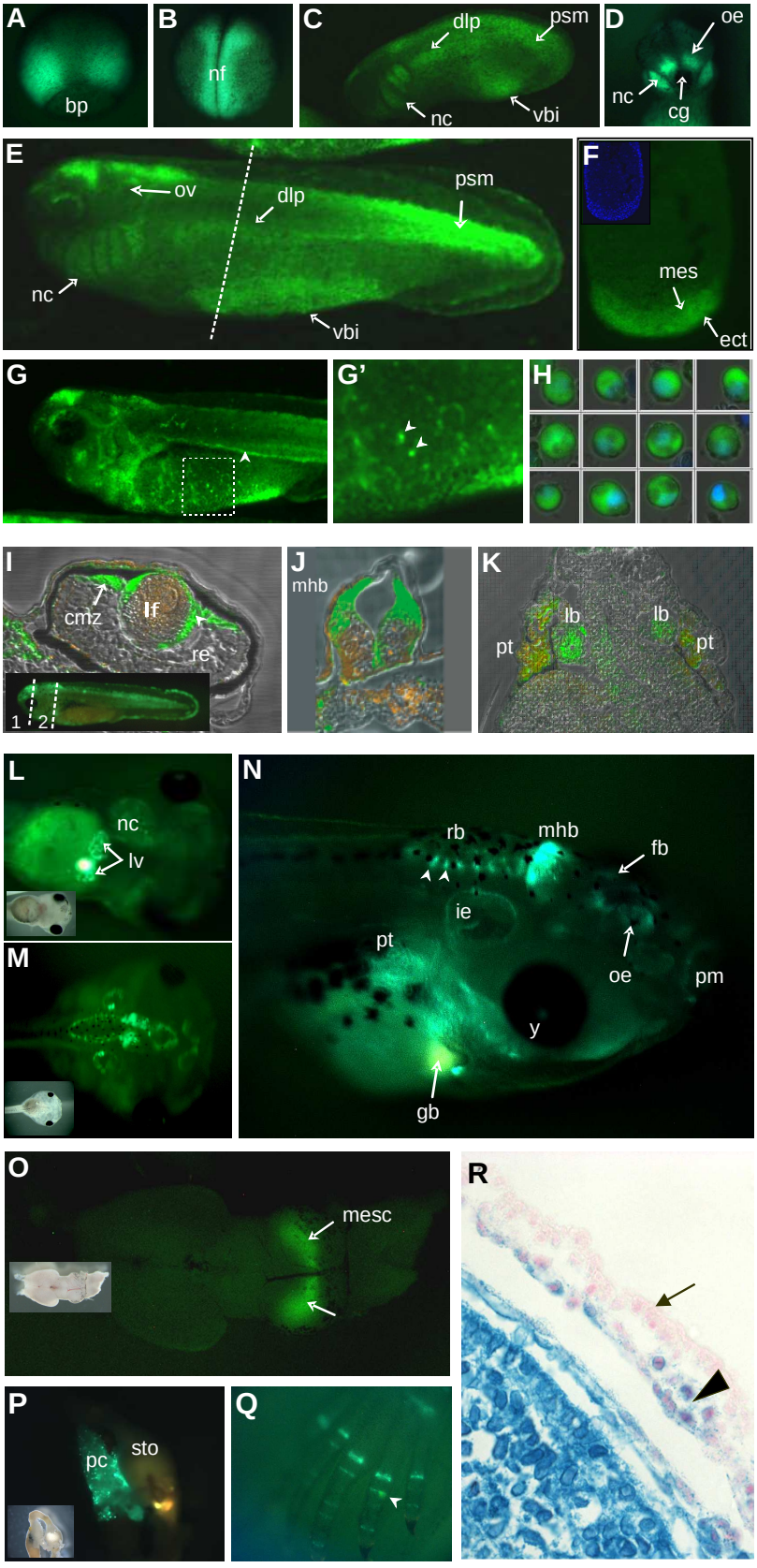
A
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Figure S2: Expression of the d2EGFP protein (A-O) in transgenic embryos carrying the Wnt/β-catenin reporter construct. (**A**) Dorsal view of a stage-11 gastrulating embryo when GFP signals first become visible. (**B-E**) Dynamic and diversified expression of GFP signal in reporter embryos as development proceeds. Particularly strong expression was observed in the developing neural folds (nf) (**B**), and in the posterior presomitic mesoderm (psm), the migrating cranial neural crest (nc), and the olfactory epithelium (oe) (**C-E**). GFP is expressed in the DLP mesoderm and the VBI, from which definitive and primitive blood cells arise, respectively (C,E). (**F**) Transverse section of a stage-32 embryo (dashed line in E) shows high levels of Wnt activity in the VBI (indicated by meso, mesoderm) and also in the overlying ectoderm (ect). The inset shows DAPI staining of the section. (**G**) Stage-37 embryos show GFP-expressing blood cells leaving the VBI through the vittelline veins and circulating in the posterior cardinal vein (arrowhead) and throughout the whole body. (**G'**) A closer view of a selected area (G; dashed rectangle) shows GFP-positive blood cells (arrowheads) circulating in the vittelline veins. (**H**) GFP-expressing and DAPI-stained individual erythrocytes collected from stage-37 embryos and viewed by confocal microscopy. (**I-K**) Confocal images of transverse sections of an early tadpole. In the inset in panel I, dashed line 1 refers to panels I and J; dashed line 2 refers to panel K. There is strong expression of GFP in the peripheral retina (cmz, I) and in the differentiating lens fibers (lf in I). Wnt activity was also detected in the lung buds (lb in K), pronephric tubules (pt in K), and the dorsal part of the midbrain-hindbrain boundary (mhb in J). (**L-N**) Views of a late tadpole stage: dorsal (L), ventral (M) and lateral (N). GFP is continuously observed in the developing liver (lv in L), pronephric tubules (pt in N), and the brain (M,N). Note that GFP is expressed at the boundaries between the rhombomeres of the hindbrain (arrowheads in N). (**O-Q**) GFP expression is visible in various organs about the time of metamorphosis, especially in the mesencephalon (mesc in O), the pancreas (pc in P), and the growth plates of the developing limbs and digits (arrowhead in Q). Insets correspond to bright field images. (**R**) Detection of Wnt/β-catenin signaling in an E8.5 embryo of the BAT-Gal Wnt-reporter mouse. Strong β-galactosidase activity is visible in the embryo proper (lower left half) and in the extraembryonic mesoderm that forms the blood islands in the yolk sac (arrowhead). No activity is seen in the surrounding visceral endoderm (arrow).

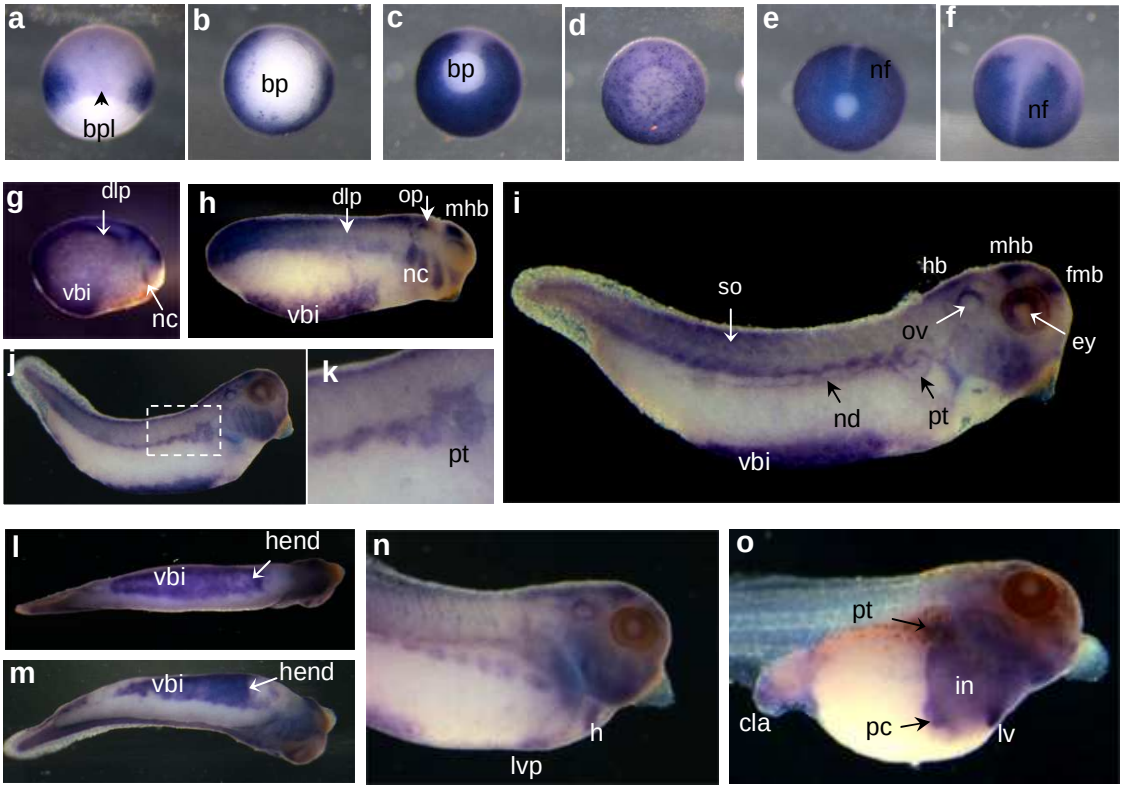

Figure S3. *In situ* hybridization shows expression of the d2EGFP transcripts in transgenic embryos carrying the Wnt/β-catenin reporter construct. **(a)** Dorsal and **(b)** vegetal views of a stage-10.5 embryo; the arrowhead indicates the absence of GFP expression at the dorsal blastopore lip. **(c)** Vegetal and **(d)** animal views of a stage-11.5 embryo (dorsal side up) show Wnt activity comparable to that observed in vivo. The signal covers the whole ventral and lateral sides, expands toward the central midline as gastrulation proceeds, and concentrates at the neural fold at the end of gastrulation (**e**, stage 12 posterior view, dorsal side up; **f**, stage 12, dorsal view, anterior side up). Lateral view of embryos at stages 21 **(g)** and 28 **(h)**: anterior side to the right, dorsal side up. Note GFP expression in the migrating cranial neural crest (nc) cells. GFP transcripts are also abundant in the DLP and VBI. **(i)** Stage-32 embryo with Wnt activity in the forebrain-midbrain (fmb) and midbrain-hindbrain (mhb) boundaries, optic vesicle (ev), otic vesicle (ov) and the posterior somites (so). **(i-k)** Dynamic Wnt activity in the nephric duct (nd); the first signal appears together with the signal in the epithelium of the pronepric tubules (pt) (**i**) and disappears at stage 32; activity intensifies at the nephric tubules as the second nerphrostome funnel forms (**j**, rectangle enlarged in **k**). **(l,m)** Ventral view of embryos at stages 30 **(l)** and 32 **(m)** showing GFP transcripts in the hepatic endoderm (hend) anterior to the VBI **(l)**; the transcripts in the hepatic endoderm disappeared when the VBI

formed a V-shape **(m).** In stage-39 **(n)** and stage-41 **(o)** embryos, Wnt activity is detected in heart and liver primodium **(n),** and in pancreas, liver and anterior part of the intestine **(o)**. ba, branchial arches; bp, blastopore; bpl, blastopore lip; dlp, dorsal lateral plate; ect; ectoderm; endo, endoderm; ev, optic vesicle; fb, forebrain; fmb, forebrain-midbrain boundary; h, heart; hb, hindbrain; hend, hepatic endoderm; ie, inner ear; in, intestine; lv, liver; lvp, liver primodium; mb, midbrain; mhb, midbrain-hindbrain boundary; nc, neural crest; nd, nephric duct; nf, neural fold; ob, olfactory bulb; oe, olfactory epithelium; ov, otic vesicle; pc, pancreas; pt, pronephric tubules; vbi, ventral blood islands.

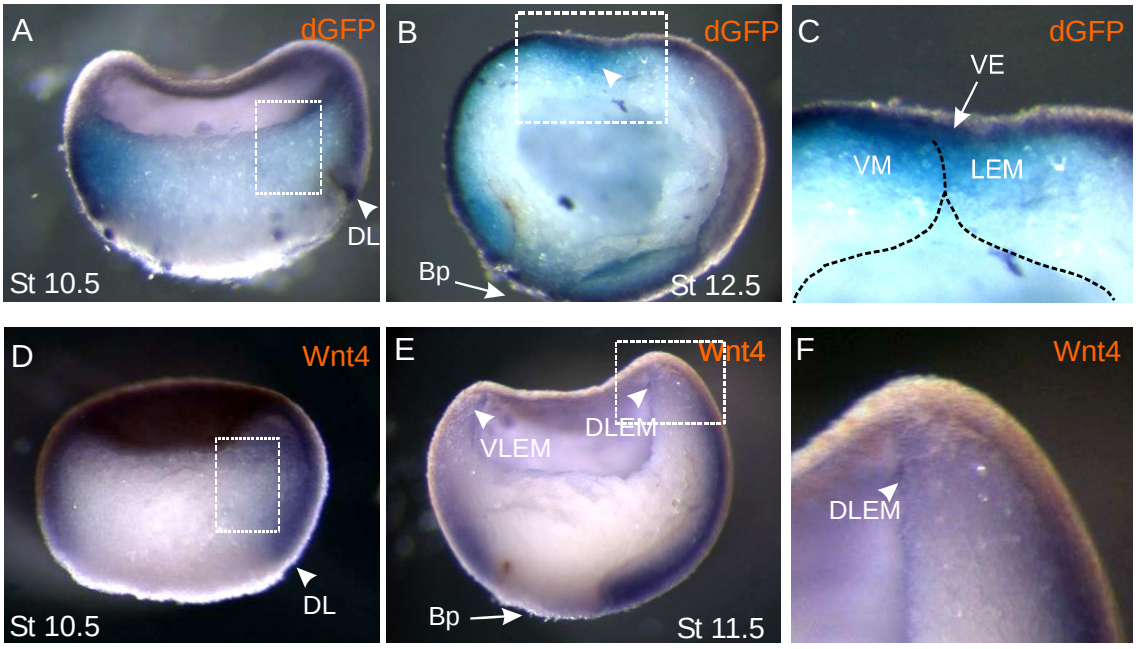

Figure S4. Expression of Wnt4 and Wnt reporter (GFP) mRNA in bisected gastrula-stage embryos. (**A-C**) WISH analysis of stage-10.5 embryos with a GFP probe showing low reporter activity in the future AEM region (dashed box) at the onset of gastrulation (**A**). At the end of gastrulation in stage-12.5 embryos, GFP transcripts are detected in the leading edge mesoderm (LEM), in the tip of the ventral mesoderm (VM) and in the overlying ventral ectoderm (VE) (the boxed area in **B** is shown enlarged in **C**). (**D-F**) Expression of *Wnt4* mRNA in bisected embryos at stages 10.5 and 11.5. At stage 11.5, *Wnt4* is expressed in the cells of the dorsal leading edge mesoderm (the boxed area in **E** is enlarged in **F**) as well as the tip of the ventral mesoderm. All embryos are shown with animal side up and dorsal to the right. Bp, blastopore; DL, dorsal lip; DLEM, dorsal leading edge mesoderm; VE, ventral ectoderm; VM, ventral mesoderm; VLEM, ventral leading edge mesoderm.

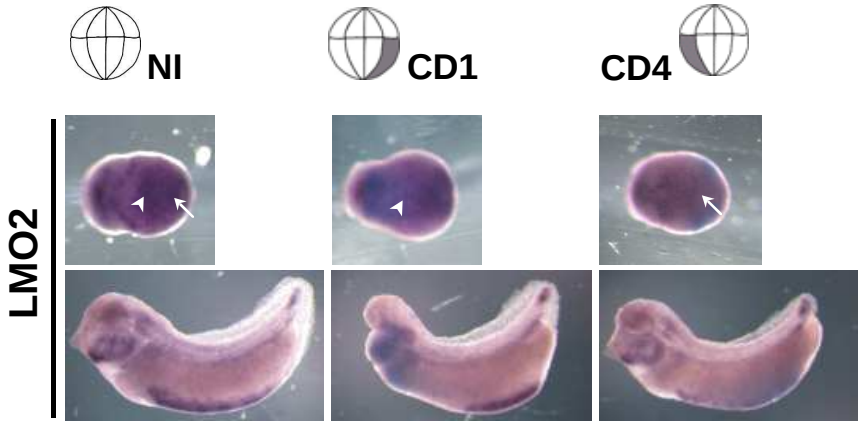

Figure S5. Depletion of Wnt4 affects the formation of the VBI (see also Fig. 3).
Targeted depletion of Wnt4 at CD1 or CD4 blastomeres (highlighted in top panel) by Wnt4 morpholino injection ablates the expression of the hematopoietic cell marker *LMO2* (violet blue) in the corresponding VBI compartments. Arrowheads and arrows indicate aVBI and pVBI, respectively. Cytoplasmic β-gal RNA was coinjected as a lineage tracer (cyan blue).

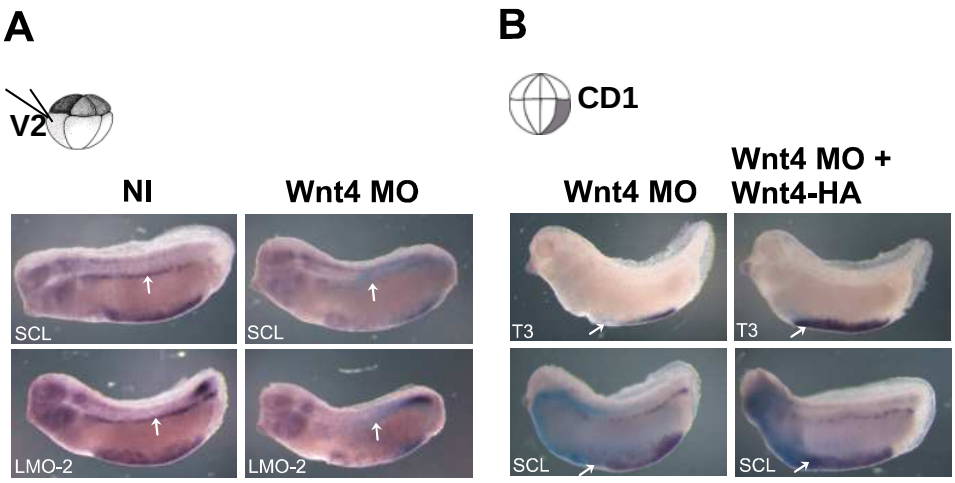

Figure S6. Effect of Wnt4 depletion on definitive blood formation and rescue experiment. **(A)** Wnt4 MO injection in V2 blastomeres of embryos at the 8-cell stage depletes the expression of the DLP markers *SCL* and *LMO2* (arrows). **(B)** Coinjection of 150 pg RNA of Wnt4-HA in the CD1 blastomeres rescues expression of *SCL* and *LMO2* in the corresponding areas (arrows) in Wnt4-depleted embryos; 10 pg RNA of cytoplasmic β-gal was coinjected as lineage tracer. The arrow shows the aVBI. The injected doses of Wnt4 MO were 2.5 ng and 4 ng for embryos at the 16-cell and 8-cell stages, respectively.

Figure S7. Wnt/β-catenin signaling is required in both ectoderm and mesoderm for the formation of the VBI (see also Fig. 4). Embryos were injected at the 16-cell stage either in the CD1, CD4 or A4 blastomere (as indicated on top) with 100 pg RNA of EnR-LEFΔN-GR and 10 pg RNA of cytoplasmic β-gal as lineage tracer. Embryos were treated with 10 µM Dex during gastrulation (stage 11.5) and fixed at stage 20/21 or 32, stained for β-gal, and then processed for WISH. *LMO2* was completely abrogated in Dex-treated embryos, regardless of the injection sites.

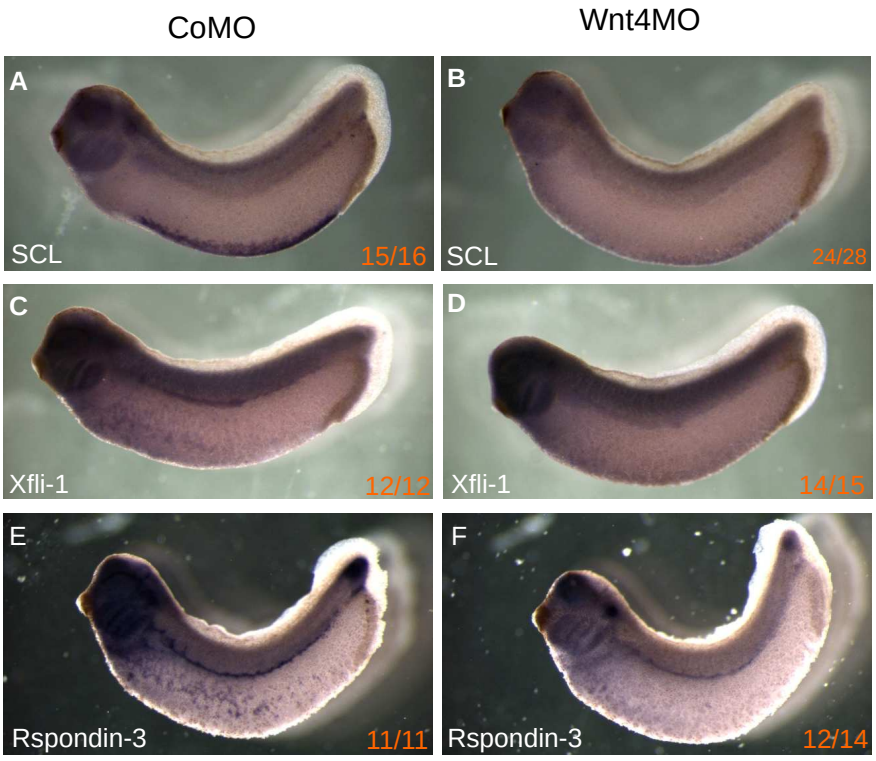

Figure S8. Expression of Xfli1 and R-spondin 3 (Rspo3) in Wnt4-depleted embryos. *X. laevis* embryos were injected with 12 ng Co MO (A, C, E) or Wnt4 MO (B, D, F) at the 4-cell stage in the dorsal and ventral marginal zones and cultured until stage 29-30. WISH analysis was performed for the hematopoietic marker *SCL* (**A, B**), the endothelial marker *Xfli-1* (**C, D**) and for *Rspo3* (**E, F**). In addition to *SCL*, which was strongly decreased in Wnt4 morphants, also *Xfli-1* and *Rspo3* were slightly reduced in the region bordering the VBI. The ratio in the right lower corner indicates the number of embryos displaying the phenotype shown in the picture.

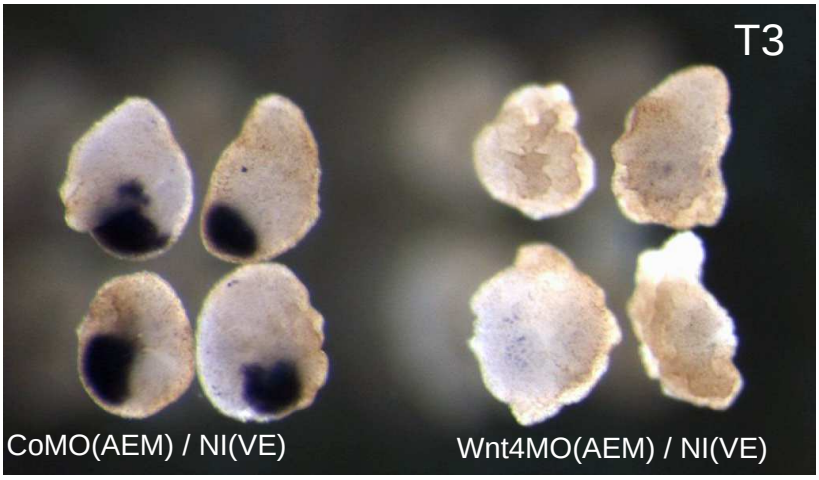

Figure S9. Wnt4 is required in the AEM for the expression of the erythroid marker *αT3-globin* (T3) in the combined explant assay. AEM of embryos injected with Co MO or Wnt4 MO were explanted and conjugated with ventral ectoderm (VE) of noninjected (NI) embryos at stage 10.5. The combined explants were cultured until stage 32 and processed for WISH. *αT3-globin* expression in the AEM was completely abolished by Wnt4 depletion.

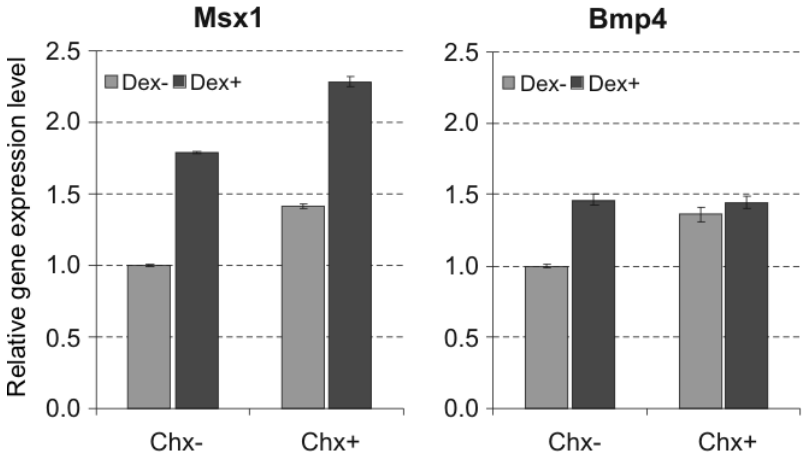

Figure S10. Identification of *BMP4* as an indirect target gene of Wnt/β-catenin signaling. Influence of the translation inhibitor cycloheximide (CHX) on Wnt-induced expression of *BMP4* and *Msx1*. Embryos were injected at the 4-cell stage in each blastomere with 25 pg inducible Wnt-activating LefΔN-VP16-GR RNA and treated at tailbud stage with Dex and/or CHX for 2 hours. While both *BMP4* and *Msx1* are induced by the Dex-mediated activation of LefΔN-VP16-GR, only *Msx1*, which is a known Wnt target gene, was induced in the presence of CHX.

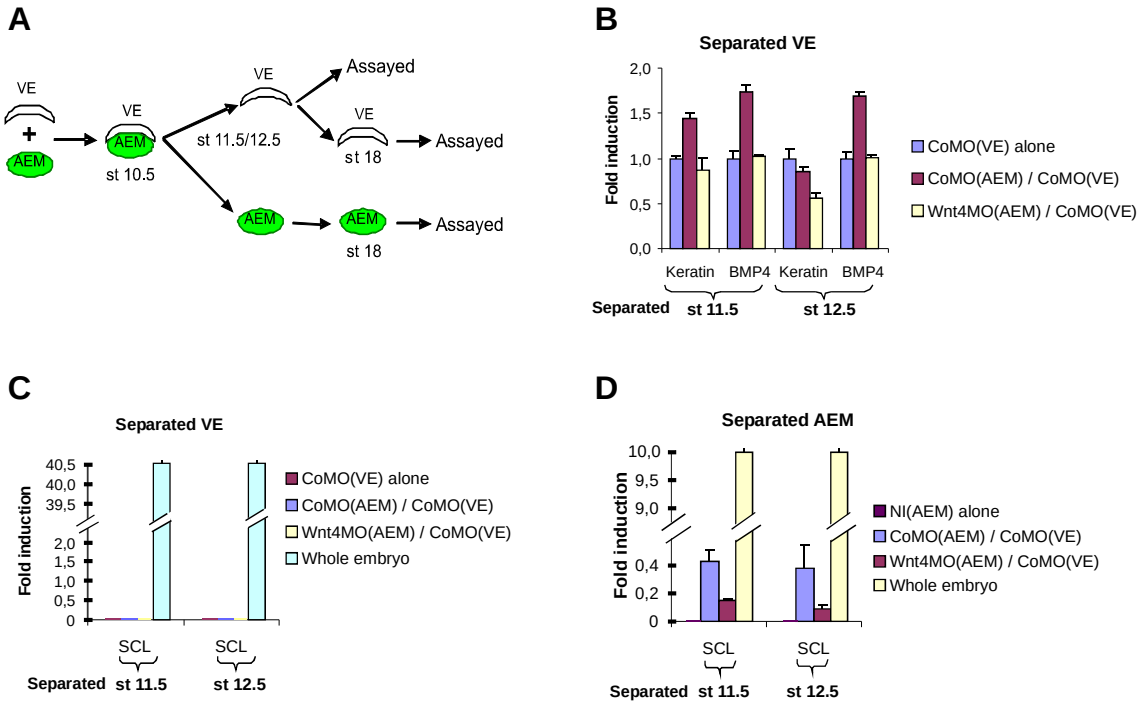

Figure S11. A short (1.5 hours) contact is sufficient for Wnt4 in the AEM to regulate BMP4 expression in the VE. (**A**) Schematic representation of the experimental setup. Embryos were injected in the marginal zones at the 2-cell stage with 12 ng Co MO or Wnt4 MO. Oregon Green 514 was coinjected in embryos that were used for AEM explants. At stage 10.5, VE and AEM were explanted, combined, and cultured for 1.5 or 3 hours to reach a state equivalent to stage 11.5 or 12.5, respectively. VE was then separated from AEM under the fluorescent microscope and then either assayed immediately by qPCR for *BMP4* and epidermal marker *Keratin* (**B**) or cultured until stage 18 and assayed for the hematopoietic marker *SCL* (**C**). VE of Co MO was cultured alone and assayed the same way. Whole embryos were used as positive control. The separated AEM was cultured until stage 18 and assayed for *SCL* expression (**D**). Uncombined AEM and whole embryos at equivalent stages were used as control. Error bars represent standard deviation.

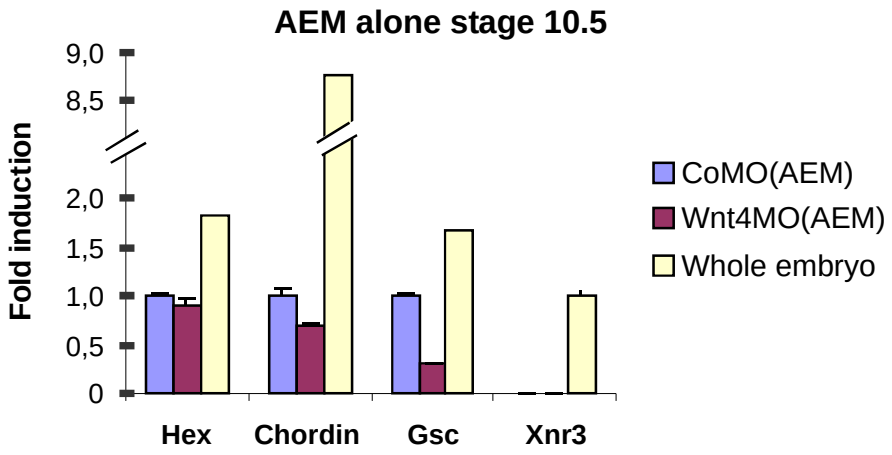

Figure S12. Spemann organizer genes are not increased in the AEM of Wnt4 morphants. Embryos were injected in the marginal zones at the 2-cell stage with 12 ng Co MO or Wnt4 MO. At stage 10.5, anterior endomesoderm (AEM) was explanted, cultured to stage 11, and assayed for expression of the organizer genes *Chordin*, *Goosecoid* (*Gsc*) and *Nodal-related 3* (*Xnr3*). An endodermal marker (*Hex*) was assayed as a reference for the total amount of endomesodermal tissue being explanted. Whole embryos at an equivalent stage were used as positive control. Error bars represent standard deviation.

Movie S1 caption:

A stage-35 *Xenopus tropicalis* tadpole transgenic for the Wnt reporter Bin8Lef-GFP, showing GFP–positive blood cells leaving the VBI and entering in the circulation network.

Table S1. Correlation of domains of GFP expression in Bin8Lef-GFP Wnt reporter embryos and tadpoles with the corresponding expression domains of Wnt ligands or Frizzled receptors.

Neurula		**Tailbud**		**Tadpole**	
Sites of reporter activity	**Expression of Wnts or Fzs**	**Sites of reporter activity**	**Expression of Wnts or Fzs**	**Sites of reporter activity**	**Expression of Wnts or Fzs**
Neural fold	Wnt1(1); Wnt3a(2); Fz3(3); Fz-10A,B(4); Wnt6(5)	Neural tube	Fz3(3)	Forebrain-midbrain boundary	Wnt3A(2), Wnt8B(6)
Prospective midbrain-hindbrain boundary	Wnt1(1); Wnt8B(6); Wnt2B(7)	Midbrain-hindbrain boundary, Dorsal midbrain-hindbrain boundary Midbrain	Wnt1(2) Fz9(8); Fz3(3) Wnt10B(9) Wnt4 (**Fig. 3F**)	Midbrain-hindbrain boundary	Wnt1(2); Wnt3(10); Wnt4 (**Fig. 3G**)
Dorsal neural tube	Wnt7B(11)	Hindbrain Dorsal hindbrain	Wnt3A(2); Wnt10B(9); Fz10A,B(4); Fz3(3); Fz9(8); Wnt4 (**Fig. 3F**) Wnt1(11)	Hindbrain Posterior hind brain	Wnt3(10); Wnt4 (**Fig. 3G**) Wnt1(2, 9, 11); Wnt8B(6)
Neural crest, (Branchial arches)	Fz2(10); Fz7(8)	Branchial arches	Wnt4(12); Wnt11(13, 14); Wnt9B(10); Wnt7B(9, 11); Fz2(10); Fz8(15)	Branchial cartilage	Wnt4 (**Fig. 3G**) Wnt6(5)
Optic anlage	Wnt6(5); Wnt7B(11); Fz3(3); Fz2(10); Fz5(16); Wnt9B(17)	Optic vesicles, eyes, CMZ	Wnt1(2); Wnt3A(2); Wnt4 (**Fig.3F**); Wnt6(5); Wnt9B(10); Wnt7B(9, 11); Fz2(10); Fz7(8); Fz3(3);Fz5(16)	Eyes	Wnt4 (**Fig. 3G**); Wnt16(10); Fz5(16)
Otic anlage	Wnt3A(2); Fz2(10)	Otic vesicle	Wnt3A(2); Wnt9B(17), Fz2, Fz3(3)	Inner ear	Wnt3A(2)
Ventral blood islands, dorsal lateral plate	Wnt4(12)	Ventral blood island, dorsal lateral plate	Wnt4(12)	Ventral blood islands	Wnt4(12)
Pronephric anlarge	Wnt4(12), Wnt6(5)	Pronephric tubules	Wnt4(12), Wnt6(5)	Pronephric tubules	Wnt4(12)
		Pronephric duct	Wnt9A(17);	Pronephric duct	Wnt9A(17)

Neurula		**Tailbud**		**Tadpole**	
Sites of reporter activity	**Expression of Wnts or Fzs**	**Sites of reporter activity**	**Expression of Wnts or Fzs**	**Sites of reporter activity**	**Expression of Wnts or Fzs**
			Fz7(8); Fz8(15, 18)		
Paraxial mesoderm	Fz2(10); Fz3(3)	Presomitic mesoderm and Somites	Wnt11(13, 14); Wnt6(5); Fz2(10)	Somites	Wnt6(5); Wnt10A(17); Fz2(10)
Epidermis	Wnt8B(6); Wnt6(5)	Epidermis	Wnt8B(6); Wnt7B(2, 11); Wnt6(5)	Epidermis	Wnt6(5), Wnt7B(9)
		Tail bud	Fz5(16)	Dorsal fin	Wnt10A(17)

Table S2. Summary of WISH marker gene expression in Wnt4 morpholino injected *Xenopus tropicalis* embryos.

Suppression of markers is indicated by the percentage of injected embryos in which marker gene expression is strongly reduced or absent in the targeted part of the VBI; n = number of embryos analyzed; NA = not analyzed.

		Suppression of VBI markers (% (n))		
Experiments	**Injected blastomere**	**SCl**	**LMO2**	**αT3-globin**
1	CD1	100 (12)	100 (15)	84.6 (13)
	CD4	100 (12)	90 (10)	100 (6)
2	CD1	92.3 (13)	100 (8)	77.8 (9)
	CD4	84.6 (13)	100 (12)	100 (15)
3	CD1	100 (14)	NA	100 (6)
	CD4	94.4 (18)	NA	100 (8)
4	CD1	87.5 (16)	NA	88.9 (9)
	CD4	88.2 (17)	NA	87.5 (8)

Embryos were injected with Wnt4MO and processed for WISH as indicated in Figure 3B. *SCL* and *LMO2* expression was analyzed in stage 21 and 32 embryos. *αT3-globin* expression was analyzed at stage 32.

Table S3. Summary of WISH marker gene expression in EnR-LEFΔN-GR injected *Xenopus tropicalis* embryos.

Suppression of markers is indicated by percentage of injected embryos with strongly reduced or absent marker gene expression at the VBI; n = number of embryos analyzed; NA = not analyzed.

		Suppression of VBI markers (% (n))		
Experiments	**Injected blastomere**	**SCL**	**LMO2**	**αT3-globin**
1	CD1	66.7 (12)	75 (12)	62.5 (8)
	CD4	85.7 (14)	91.6 (12)	85.7 (7)
2	CD1	90 (16)	73.3 (15)	71.4 (7)
	CD4	87.5 (19)	93.75 (16)	100 (5)
	A4	75 (8)	71.4 (7)	75 (4)
3	CD1	75 (20)	NA	66.7 (9)
	CD4	94.7 (19)	NA	87.5 (8)
	A4	70 (10)	NA	66.7 (6)

Embryos were injected with EnR-LEFΔN-GR RNA and processed for WISH as indicated in Figure 4C. SCL and LMO2 expression was analyzed in stage 21 and 32 embryos. *αT3-globin* expression was analyzed at stage 32.

Description of Figures S1-3 and Table S1.

We designed and characterized a new reporter construct consisting of a synthetic promoter containing eight copies of an optimal binding sequence for LEF/TCF factors and a minimal TATA box in front of a reporter gene **(Fig. S1)**. Analysis of this promoter in cell lines showed that its activity is comparable to that of the most frequently used pTOP-TK reporter (pTOP-Flash) {Korinek, 1998 #28} **(Fig. S1A)**. To reveal as clearly as possible the temporal dynamics of Wnt pathway activation, we used destabilized EGFP as a reporter gene and flanked it with chromosomal insulator sequences from the chicken β-*globin* gene to increase its expression {Sekkali, 2008 #29}. Cotransfection of this reporter construct with a stabilized form of β-catenin can induce expression of GFP, which confirms that addition of insulators does not change the potential activity of the promoter **(Fig. S1C)**. The reporter construct (p8Lef-dGFP) was integrated by transgenesis in *X. tropicalis* embryos.

More than half of the embryos generated with our optimized reported construct expressed GFP. In the several F_0 lines that were generated, the reporter was transferred to the successive generations. F_1 embryos treated with LiCl, which stabilizes β-catenin, showed ectopic expression of GFP transcripts in the whole embryo (**Fig. S1D),** whereas the non-treated transgenic siblings had a defined pattern. We further tested if the interference at the level of β-catenin transcriptional activation could affect the expression pattern of GFP. We injected the inducible Wnt-interfering construct, pEnR-LEFΔN-GR, either in the dorsal or the ventral side of four-cell stage embryos. This Wnt-interfering construct had been generated and characterized previously {Deroo, 2004 #31}. In response to Dexamethasone (Dex), it can bind to LEF-1/TCF transcription factor binding sites at the promoter of β-catenin target genes and repress their transcription {Deroo, 2004 #31}. Injected embryos treated with Dex showed loss of GFP signal in the area corresponding to the injection site (**Fig. S1E**).

Expression of GFP was analyzed throughout embryonic development and larval growth. Both fluorescent illumination and whole mount in situ hybridization (WISH) with a GFP probe showed several recurrent patterns of GFP expression in the transgenic embryos and the growing tadpoles (**Figs. S2**, **S3 and Table S1**). Below we present a full description of these dynamic GFP expression patterns and a description of correlated expression domains of Wnt ligands and Frizzled receptors or reported biological functions of the canonical Wnt pathway during early and late development (see **Table S1** and discussion below). In summary,

we obtained several transgenic lines that faithfully report the activation of the canonical Wnt pathway in both the embryonic and juvenile stages.

Expression of GFP in transgenics could first be detected clearly in the ventral region of gastrula stage embryos, but it was absent from the most dorsal region (**Fig. S2A** and **Fig S3a-b**). This GFP pattern reflects the first known zygotic Wnt signal mediated by Wnt8 {Christian, 1993 #22}. As gastrulation proceeded, the GFP signal persisted in the Wnt8 domain (**Fig. S2A** and **Fig. S3a-c**) and became stronger at the borders of the neural plate, where it was also evident at the domain of arising neural crest, but it was absent from the most anterior side of the embryo (**Fig. S3f**). At the neurula stage, the signal was enriched at the neural folds (**Fig. S2B**), where some Wnt ligands and receptors, e.g. Wnt3a and Frz3, are expressed{Gradl, 1999 #32}, and expanded further to the whole epidermis.

It is important to note that GFP was totally absent from the central anterior part of the prospective neural tube in neurula stage embryos (see **Fig. S2B** and **Fig. S3f**). The absence of a Wnt-reporter signal is in agreement with previous data showing high expression levels of antagonists of Wnt/β-catenin signaling (GSK-3β and TCF3) in this area during neurulation{Onai, 2004 #60}. Absence of anterior Wnt signaling is required for the correct anterior-posterior patterning of the developing brain of *Xenopus* embryos. Moreover, this anterior region might still be under negative control of the Wnt antagonists sFRP/Frzb, Dkk and Cerberus, which are secreted by the endomesoderm underlying the neural plate{Yamaguchi, 2001 #61}. Thus, our reporter embryos provide additional evidence for the role of Wnt/β-catenin signaling in the patterning of the anterior-posterior axis of the *Xenopus* brain.

At the tailbud stage, GFP signals appeared in the future midbrain-hindbrain boundary, and the pronephric mesoderm (**Fig. S2C** and **Fig. S3h**). Again, these signals reflect the expression of several Wnt ligands, e.g. Wnt1 and Wnt3a (midbrain-hindbrain boundary, posterior hindbrain){Gradl, 1999 #32}, Wnt4 (midbrain, hindbrain and pronephros){McGrew, 1992 #24}, Wnt2 and Wnt2B (midbrain-hindbrain boundary){Gradl, 1999 #32}. GFP expression was also detected in the migrating cranial neural crest cells, which express Wnt11r{Matthews, 2008 #33}, and in the otic and optic vesicles, which express Frz3{Rasmussen, 2001 #34}. Different sites of neural crest cells were also marked with strong Wnt activity (see **Fig. S2C-E** and **Fig. S3g-i**). An inductive role for Wnt signaling in neural crest (NC) formation is supported by various studies in *Xenopus*{Ciani, 2005 #62}, and Wnt/β-catenin signaling in the hindbrain is responsible for the generation of neural crest cells

and their migration to the branchial arches. Interestingly, we observed that the fifth branchial arch was the result of the splitting of the fourth arch, which has never been reported before. From neurula until late tailbud stage, GFP was also expressed weakly in the epidermis, where Wnt6 was recently reported to be expressed{Lavery, 2008 #37}

At the early tadpole stage (**Fig. S2E, Fig. S3i-j**), additional signals likely reflect the activity of Wnt signaling associated with expression of Frz5 at the dorsal retina{Sumanas, 2001 #35}, Wnt3a in the forebrain-midbrain boundary and dorsal otic vesicle, and Wnt8b in the forebrain-midbrain boundary and posterior midbrain{Gradl, 1999 #32}. Wnt7B was reported to be expressed in the dorsal roof plate of the neural tube and in the epidermis{Gradl, 1999 #32; Wolda, 1992 #36}. Also, Fz9 is specifically expressed at the dorsal side of the neural tube at the tadpole stage{Gradl, 1999 #32}. As somitogenesis proceeded, GFP expression gradually increased in the presomitic mesoderm and the posterior somites and faded in the anterior direction (**Fig. S2C and S2E)**. Cryosectioning of early tadpoles further showed expression in the peripheral region of the retina, the lens, the dorsal side of the hindbrain, and the lungbuds **(Fig. S2I-K)**.

At the late tadpole stage, GFP was clearly detected in specific regions of the brain, including rhombomere boundaries, craniofacial cartilage, kidney, liver and pancreas (**Fig. S2L-N** and **Fig. S3n-o**). It has been shown that Wnt family molecules in the developing peripheral and central nervous systems regulate diverse cellular events, including the patterning of the anterior-posterior and dorsal-ventral axes, cell proliferation and fate determination, cell polarity and movement, axonal growth, and programmed cell death{Ciani, 2005 #62}. Wnt ligands, such as Wnt-1, Wnt2b, Wnt-3a, Wnt-8b and Wnt4{Gradl, 1999 #32; Lyons, 2004 #26; Wolda, 1992 #36}, which function via the canonical Wnt pathway and the Wnt target gene En-2{McGrew, 1999 #38}, were previously reported to be active in the midbrain-hindbrain boundary, a region that was also detected by the Wnt-reporter systems in zebrafish{Dorsky, 2002 #39} and mice{Maretto, 2003 #40}. In the hindbrain of late tadpoles, a defined pattern of GFP signal is located at the boundary of the rhombomeres (see **Fig. S2M,N)**. Studies in zebrafish have shown that Wnt and β-catenin have to be kept in balance to restrict the boundary and prevent its expansion{Amoyel, 2005 #63}. In sum, we observed the expression pattern of GFP in the neural tube derivatives in Bin8Lef-GFP embryos throughout different stages of development, reflecting the spatiotemporal requirements for Wnt/β-catenin signaling in the developing neural tissues.

Close to metamorphosis, restricted zones of GFP expression were prominent in the growth zones of bones, the midbrain, and the dorsal pancreas **(Fig. S2O-Q).**

The pattern of the reporter gene in transgenic embryos also documents the dynamic temporal activity of endogenous Wnt signaling during organogenesis. For example, the GFP pattern in situ changed during liver development, which indicates that Wnt signaling is required at defined developmental stages{McLin, 2007 #64; Goessling, 2008 #65} (see **Fig. S3n** and **S3o**). Likewise, mounting evidence suggests that timed canonical Wnt/β-catenin signals are also critical in kidney development{Lyons, 2009 #66} and segmental patterning in *Xenopus* somitogenesis{Wang, 2007 #67}. Furthermore, Wnt/β-catenin signaling is active in undifferentiated somites in the mouse{Maretto, 2003 #40}, and a nuclear β-catenin gradient is observed in the posterior region of presomitic mesoderm controlling key aspects of segment formation{Aulehla, 2008 #68}. Our finding (see **Fig. S2C** and **1E**) supports the hypothesis that this controlling mechanism might also be conserved in *Xenopus*.

As already mentioned, many signals can be directly linked to known expression and activities of specific Wnt ligands during organogenesis and tissue formation (see **Table S1** for an overview). For most of the observed domains, direct or indirect supporting evidence can be extracted from the literature. The canonical Wnt pathway has multiple roles during eye development, as illustrated by expression of many Wnt ligands and Fz receptors in the lens, neural retina and retinal pigmented epithelium{Van Raay, 2004 #41; Van Raay, 2005 #42}. The Wnt ligands Wnt1, -3a and -8, which are present in the neural plate, have been shown to play an important role in specifying the otic placode in *Xenopus* embryos by directing its subsequent development into an otic vesicle{Park, 2008 #43}. Developing somites express several Fzs{Wheeler, 1999 #44}, and the canonical Wnt/β-catenin pathway has been demonstrated to regulate myogenesis through R-spondin2 in *Xenopus* embryos{Kazanskaya, 2004 #45}.

Importantly, Bin8Lef-GFP embryos revealed new sites of β-catenin activity in regions in which the role of Wnt/β-catenin signaling has not been documented, e.g. in the lung buds (see **Fig. S2K**), the ventral blood island, the growth zone of developing limbs (see **Fig. S2Q**), and the dorsal pancreas (see **Fig. S2P**). Detailed observation of Bin8Lef-GFP in embryos and/or frogs might uncover other Wnt/β-catenin activation centers.

Our Wnt-reporter construct is based on multiple Lef1/TCF binding sites combined with a minimal promoter TATA box and destabilized GFP as the reporter. Insulator elements

were incorporated to overcome mosaic expression due to variation and variegation as well as to enhance expression of the reporter gene. Our Wnt-reporter construct contains several features that make it superior to other Wnt-reporters used previously in *Xenopus*{Denayer, 2005 #49; Geng, 2003 #50} and in other transgenic animal models, including zebrafish{Dorsky, 2002 #39} and mouse{Maretto, 2003 #40}. Minimal promoters, e.g. c-fos, have been used in a Wnt-reporter construct for *Xenopus*, but this construct failed to label sites of known Wnt/β-catenin signaling{Geng, 2003 #50}. The use of a minimal TK promoter was more successful{Denayer, 2005 #49}, which emphasizes the possibly pivotal importance of the identity of the minimal promoter in correctly addressing the signaling activity. In the mouse, TOP-gal (containing a c-fos minimal promoter) failed to reveal β-catenin activity in the dermal papilla{DasGupta, 1999 #51}, a known Wnt/β-catenin signaling centre, which instead was clearly labeled in BAT-gal (containing a siamois minimal promoter) mice{Maretto, 2003 #40}. Our reporter constructs contain a minimal TATA box as a promoter element downstream of eight copies of the LEF-1/TCF binding site. We observed in transfection assays that p8Lef-luciferase had a much higher signal-to-noise ratio than the conventional pTOP-flash, which contains a minimal TK promoter. As a reporter gene we used destabilized GFP, which is ideal for analyzing the dynamic activity of a signaling pathway in vivo in rapidly developing *Xenopus* embryos. Destabilized GFP has an inherently low level of expression, but this was overcome by flanking the transgene with tandem copies of the chicken β-globin insulator. Indeed, by combining the above-mentioned elements in the final reporter construct (Bin8Lef-GFP) we were able to generate many transgenic embryos in which consistent GFP expression patterns could be detected. The signal generated in transgenic embryos was specific for Wnt/β-catenin signaling, as shown by its specific responses to different factors that interfere with the signaling activity.

Importantly, we showed that transgenic embryos generated from the Bin8Lef-GFP construct reflect all known domains of Wnt/β-catenin signaling activity during early development of *Xenopus* (**Fig. S2 and S3)**. However, some words of caution are appropriate. Lef1/TCF-mediated gene activation might result from a combination of various signaling inputs in a certain cellular context. Signals other than Wnts, e.g. TGF-β signaling, might induce β-catenin stabilization{Jian, 2006 #52}. Conversely, signaling outputs from other cofactors for nuclear β-catenin rather than Lef1/TCF would not be detected. Despite these potential shortcomings, our transgenic *Xenopus* Wnt-reporter lines have great potential for further investigation of the role of the Wnt signaling pathway and for uncovering novel functions in both embryonic and adult tissues and organs.

Supplemental Materials and Methods

Plasmid construction

We amplified 8 x LEF-1/TCF binding sites and 8 x mutated sites by PCR from pSuperTOPflash and pSuperFOPFlash (gift from Randall Moon), respectively, with primer pairs 5'-ACGCGTCGACGGTACCGAGCTCTTACGC-3' and 5'-CGGGATCCTTTACCAACAGTACCGGAATG-3', and cloned them between the *Sma*I and *Bam*HI sites of pd2EGFP-control (Clontech, Inc.). The fragments were then excised with *Sal*I and *Hin*dIII and cloned into pCS2+ cut with *Sal*I and *Hin*dIII to generate p8lef-GFP and p8mLef-GFP. 8Lef-GFP and 8mLef-GFP were then excised as *Kpn*I-*Not*I fragments, blunt-ended, and cloned into pbinV2{Sekkali, 2008 #29} at *Kpn*I-*Eco*RV to produce pBin8Lef-GFP and pBin8mLef-GFP. To add another reference gene for identifying transgenic animals, phEc-Dsred was cut from phEc-DsR{Deroo, 2004 #31} as a *Kpn*I(blunted)-*Spe*I fragment and cloned into pBin8mLef-GFP at *Eco*RV-*Spe*I. The transgene cassettes were then released from the vector backbone with *Not*I for use in transgenesis

Cryotome, vibratome and bisections

Bin8Lef-GFP embryos were fixed in 2% formaldehyde, washed with PBS, embedded in 30% sucrose overnight, and then in cryo-embedding compound. They were cryo-sectioned at 10-μm thickness. Wnt4-WISH embryos were embedded in 5% agarose and vibratome-sectioned at 20-μm thickness. Embryos used for bisection were embedded in 3% low melting point agarose and bisected with a sharp scalpel knife.

Cell culture and transfection assay

293T cells were grown at 37°C in L-15 medium supplemented with 10% FCS (fetal calf serum), 2 mM L-Gln, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and seeded in 6-well plates. Transient transfections were done using Fugene 6 Transfection Reagent (Roche Applied Science).

For luciferase assays, the reporter constructs were cotransfected with a β-galactosidase plasmid for normalization. Constructs were cotransfected either with pCS2- β-cateninSA (gift from Gumbiner) containing *Xenopus* β-catenin with a point mutation at Serine 33, or with pCS2-Lef1 containing a human LEF1 gene. Cells were harvested after 48 hours. Lysates were prepared using Reporter Assay Lysis Buffer (Roche Applied Science) and split in triplicate. Each 25 μl of lysate was combined with 100 μl βgal substrate provided with the Galacto-Star

kit (Tropix), or with 40 µl luciferase substrate (40 mM Tricine, 2.14 mM $(MgCO_3)4Mg(OH)_2$, 5.34 mM $MgSO_4$, 66.6 mM DTT, 0.2 mM EDTA, 521 µM coenzyme A, 734 µM ATP, and 940 µM luciferin). β-gal and luciferase expression data were obtained in triplicate using the luminometer (Roche). Non-transfected cells were used as a negative control.

Explant assays and quantitative PCR analysis

For explant assays, *X. laevis* embryos were injected as indicated and cultured in 0.1x MMR until stage 10+. Ventral ectoderm and anterior endomesoderm explants were then cut in 0.5x MMR buffer using an eyebrow needle. Appropriate combinations of explants were cultured for 10 min before being transferred as a "sandwich" to a new culture dish. Explant combinations and whole embryo controls were subsequently cultured from stage 11 in 0.3x MMR in the presence or absence of hormone until harvesting at stage 15 for RNA extraction. Ten explant combinations were pooled for each RNA sample. Total RNA was extracted using Trizol reagent (Life Technologies). One microgram of RNA was used for cDNA synthesis. qPCR analysis was performed in triplicate using the qPCR kit SYBR Green (AB Biosystem) on the LightCycler 480 (Roche). The specificity of each amplicon was checked by melting curve analysis. The results were normalized with the housekeeping genes *ODC* and *EF1α.* All primers were designed using Primer Express 1.0 software (Perkin-Elmer Applied Biosystems) and are shown in Table S4.

Table S4 List of primers used for qPCR experiments

Genes (ref.)	Accession No.	Primer sequences
BMP4	NM_001088032	F 5'-CAATGAGCTCTTGCGGGATT-3'
		R 5'-GCATATAAGCGGGAACCACC-3'
Chordin		F 5'-ACGTGTACCAGCTGCCTTCC-3'
		R 5'-CTTCGGAAAGACCCCAGAGC-3'
EF1α	NM_001088052	F 5'-GCTGGAAGCTCTTGACTGCATT-3'
		R 5'-CCAATACCGCCAATTTTGTAGAC-3'
Goosecoid (48)		F 5'-TTCACCGATGAACAACTGGA-3'
		R 5'-TTCCACTTTTGGGCATTTTC-3'
Hex (49)		F 5'-ATTTCCCTGTGGGTTCTCCT-3'
		R 5'-AGGCCAGTCAGCGACTACA-3'
Keratin (50)		F 5'-CACCAGAACACAGAGTAC-3'
		R 5'-CAACCTTCCCATCAACCA-3'
Msx1		F 5'-CCTCATGGCCGATAGGAAAC-3'
		F' 5'- GATCCCACCCTGGGACTGT-3'
ODC	NM_001086698	F 5'-TACGTCAATGATGGAGTGTATGGA-3'
		R 5'-CTCATCTGGTTTGGGTTTCTTTGT-3'
SCL	NM_001088277	F 5'-CCATGCTCTATGGGCTCAATC-3'
		R 5'-AAGGTGTCTGGGTCACCAAAGT-3'
Wnt4	NM_001087728	F 5'-TGAGGAAGAGACGTGCGAAA-3'
		R 5'-GAGCACCTCGACGGACTGAA-3'
Xnr3 (49)		F 5'-AGCTCAGCCAACTTCAGCCTC-3'
		R 5'-GTTTCCCCAATTCATGATGC-3'

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