Methods.

Zebrafish husbandry, microinjections and heat shock. Zebrafish strains AB*, *Tg(TOP:GFP)*^{w25}, *Tg(-6.0itga2b:eGFP)*^{la2}, *Tg(hsp70l:Gal4)*$^{1.5kca4}$, *Tg(UAS:myc-Notch1a-intra)*^{kca3}, *Tg(tp1-MmHbb:EGFP)*^{um14}, *Tg(tp1-MmHbb:Kaede)*^{um15}, *Tg(cmyb:EGFP)*^{zf169}, *Tg(kdrl:RFP)*^{la4}, and *dlc*$^{tit446/tit446}$ (S. Holley) were maintained, crossed, injected, raised, and staged as described[30], and in accordance with IACUC guidelines. The new line *Tg(tp1-MmHbb:Kaede)*^{um15} was generated by Tol2-mediated transgenic insertion of a transgene containing a previously described Notch-responsive promoter[31] driving *Kaede* expression. Heat shocks were performed at the times indicated for 45 mins. at 37°C, as previously described[20].

Cloning, constructs, probes. Both zebrafish *wnt16* isoforms (*wnt16-001* and *wnt16-002*, Zebrafish Information Network (ZFIN)) were amplified from AB* embryo cDNA at tailbud, 9-somite, and 24 hpf, using primers (5'UTR-Wnt16-F CAGGTGCTACATATTAGATGCAGTGG, Wnt16-var-F GACATGGATAATACCGGTTGTGGG, and zWnt16-R TTACTTGCAGGTGTGCATGTCATTC) designed based on GenBank sequences NM_207096 and CD751181.1 and cloned to **pCRII**-TOPO-TA (Invitrogen) according to the manufacturer's instructions. Sequenced clones conform to since deposited GenBank sequences NM_001100046.1 (*wnt16-001*) and BC066432.1 (*wnt16-002*), with no non-silent alterations. The *wnt16-001* form was re-cloned using the primers ggatccGACATGGATAATACCGGTTGTG and ctcgagTTACTTGCAGGTGTGCATGTC to introduce 5' BamH1 and 3' Xho1 sites. *Wnt16-001* and *wnt16-002* sequences were subcloned to **pCS2+**[32] via BamH1/Xho1 or EcoR1 respectively. Full-length *dlc* was amplified from 16-ss AB* cDNA using the primers, dlc-F (ctcgagAAGATGGCTCGTGTTTTATTAAC) and dlc-R (tctagaCTATACCTCAGTAGCAAACACACG), TOPO-TA cloned, confirmed by sequencing, and subcloned to **pCS2+**. **pCS2+** *wnt3*, **pCS2+** *dnfgfr1-eGfp* (K. Poss), and **pBS** *chd* (D. Kimelman) were described previously[14]. The following probe and expression constructs were gifts as indicated: **pCS2+** *runx1* (C. Burns), **pBK-CMV** *scl* (L. Zon), **pBK-CMV** *cmyb* (L. Zon), **pBS** *flk1* (D. Ransom), **pBS** *gata1* (D. Ransom), **pCS2+** *gata2* (B. Paw), **pCRII** *rag1* (N. Trede), **pBS** *dlc* (J. Lewis), **pBS** *dld* (B. Appel), **pCS2+** *dld* (S. Holley), **pBS** *jag1b* (M. Pack), **pBS** *jag2* (M. Pack), **pCR-Script** *notch1a* (J.

Campos-Ortega), **pCR-Script** *notch1b* (M. Lardelli), **pCR-Script** *notch2* (B. Appel), **pCR-Script** *notch3* (M. Lardelli), **pSPORT1** *etsrp* (S. Sumanas), **pBS** *nkx2.2* (R. Karlström), **pCRII** *gli1* (R. Karlström), **pBS** *ptc2* (R. Karlström), **pBS** *ptc1* (J. Waxman), **pBS** *prdm1a* (J. Waxman). The following probe constructs were amplified *de novo* and cloned to **pCRII**-TOPO-TA (Invitrogen) using primers as indicated: **pCRII** *myod* (myod-F AAGATGGAGTTGTCGGATATCC, myod-R AGAATTTTAAAGCACTTGATAAATGG), **pCRII** *cdh5-frag* (cdh5-probe-F TGCCTCCGACAAGGATGAAA, cdh5-probe-R ACCGAGGTCCCCACTCATGT), **pCRII** *cdh17/cb903* (cb903/cdh17-F GCGGATGATACAGGAACAGG, cb903/cdh17-R CTGAAGGCAGATGAAGCCC), **pCRII** *col2a1a-frag* (col2a1a-probe-F CCACCTGGATTGACTGGACC, col2a1a-probe-R GTAGTGCTTGCATGTTCGGTC), **pCRII** *vegfaa*$_{165}$ (vegfAa-long-F GTTAATTTTAGCGGATTCGACG, vegfAa-short-R GATCATCATCTTGGCTTTTCAC), **pCRII** *shha* (Bam-shha-F ggatccAAAATGCGGCTTTTGAC, shha-R1-R gaattcTCAGCTTGAGTTTACTGACATCC; subsequently subcloned to **pCS2+**), **pCRII** *foxc1a* (foxc1a-F GTCATGCAGGCGCGCTATT, foxc1a-R ctcgagTCAAAATTTGCTGCAGTCATACAC), **pCRII** *foxc1b* (foxc1b-F ggatccACGATGCAGGCGCGCTACCC, foxc1b-R TCAGAACTTGCTGCAGTCGTATAC), **pCRII** *twist1b* (twist1b-F GAGATGCCCGAAGAGCCCGCGC, twist1b-R ctcgagCTAGTGAGATGCAGACATGGACC), **pCRII** *twist2* (twist2-F GAAATGGAAGAGAGTTCTAGCTC, twist2-R ctcgagCTAGTGGGACGCAGACATCG). A *pax1* fragment corresponding exactly to the 1080 bp ORF found in NM_001080592, which has been annotated as the zebrafish *Pax1* orthologue at NCBI Homologene (HomoloGene:4514) was amplified (R1-pax1-F gaattcAAAATGCTTTCGTGTTTTGCAGAG, pax1-Xba-R tctagaTTACGAGGATGAGGTAGAAAGGC) from 24 hpf AB* cDNA to generate **pCRII** *pax1*. The *pax1* gene is located on chromosome 17 and shows syntenic conservation of the 5' neighbor (*Nkx2.2* in mouse and *NKX2.2* in human). The encoded protein is 69% identical and 90% similar to mouse and human Pax1. A 5' Xho1/Cla1 fragment of **pBS** *efnb2a* (gift of C. Moens) was recloned to **pBS** to generate **pBS** *efnb2a-probe*. Digoxigenin- and fluorescein-labelled probes were generated as described

previously[33] using 5' cut sites and RNA polymerases as appropriate (details available on request). Embryos were mounted and photographed as described[14].

mRNA, morpholinos and injections. 5'-G-capped mRNAs were synthesized from Not1- or Asp718- (**pCS2+** dlc) linearized **pCS2+** constructs as described previously[14], using the mMessage mMachinge kit (Ambion, Austin, TX). The following morpholino antisense oligonucleotides were synthesized by Gene Tools, LLC (Philomath, OR) and suspended as 25 mg/ml stocks in DEPC ddH_2O and diluted to injection strengths: W16MO1 AGGTTAGTTCTGTCACCCACCTGTC, W16MO2 GCGTGGAATACTTACATCCAACTTC, W16CoMO2 ("CoMO" in the text) AcGTTAGTTgTGTCAgCCAgCTcTC, dldMO2 AAACAGCTATCATTAGTCGTCCCAT[19], W11MO GAAAGTTCCTGTATTCTGTCATGTC[34]. Injections were performed as described previously[14], and 0.1% phenol red (Sigma Aldrich, St. Louis, MO) was included as an indicator. W16MO1 and W16MO2 were injected individually at 5ng and combinatorially at 2 ng W16MO1 plus 3 ng W16MO2. In all depicted cases, both morpholinos caused the representative phenotypes shown, thus embryos are labelled "W16MO". 5 or 7 ng of dldMO and W11MO were injected. 50 pg of *dlc* and/or *dld* mRNA were injected for rescue experiments.

Genomic and phylogenetic analyses. Alignments, genomic analyses, and phylogenetic comparisons were performed as described previously[14] using the following sequences Wnt16l NP_001093516.1, mWnt16 NP_444346.3, hWNT16a NP_057171.2, hWNT16b NP_476509.1, and hWNT4 NP_110388.2 (as the outgroup). Sequencing analysis was performed with Sequencher software (GeneCodes Corp., Ann Arbor, MI).

PCR Genotyping and RT-PCR. Fixed, WISH-processed individual embryos had DNA isolated in lysis buffer (10mM Tris, pH 8.3, 50mM KCl, 0.3% Tween-20, 0.3% NP-40), 98°C, 10 minutes, held at 4°C to allow addition of proteinase K to a final concentration of 1 mg/ml, 18 hours at 55°C, 15 minutes at 98°C. Presence of the *UAS:NICD* transgene was assessed by PCR using the primers E1B-F CATCGCGTCTCAGCCTCAC and Notch-R CGGAATCGTTTATTGGTGTCG (T_m 55°C, extension time 45 sec., 35 cycles), with *ef-1α*-F GTGCTGTGCTGATTGTTGCT, *ef-1α*-R

TGTATGCGCTGACTTCCTTG as a positive control (T_m 56°C, extension time 30 sec., 25 cycles). For RT-PCR, RNA was isolated from groups of 30 whole embryos at the stages indicated, and cDNA prepared as described previously[14]. PCR on cDNA was amplified using the primers, *ef-1α* (as above), *wnt16*-RT1-F ACTAAAGAGACAGCGTTCATCC, *wnt16*-RT1-R AACTCATCTTTGGTGATAGGC, *wnt16*-RT3-F TTGTGGGATACATGCAGTTCA, and *wnt16*-RT3-R CACAGCTCCTTCTGCTTGTG with Taq polymerase (Invitrogen, Philadelphia, PA) at a T_m of 56°C, extension times of 30 sec., 38 cycles. Gels were imaged as described[14].

Whole mount in situ and antibody staining. Single and double enzymatic WISH was performed as described previously[33]. Double fluorescence in situs were performed according to published protocols[18]. Whole mount immunofluorescence was performed as described[35], using anti-Myc monoclonal 9E10 antibodies at 1:200 (Covance, Princeton, NJ) and Dylight488 AffiniPure Donkey Anti-Mouse IgG secondary (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:100.

Confocal, fluorescence microscopy, photoconversion, and timelapse imaging. Confocal images were acquired, essentially as described[7]. Photoconversion of Kaede proteins was performed using the Leica SP5 ROI and bleach functions on whole embryos, excluding heads, using a 405 nm laser line for 2 minutes. Kaede fluorescence was visualized using 488 nm and 543 nm laser lines. Fluorescence images of transgenic animals were acquired on a Leica DMI 6000 (Leica Microsystem, Bannockburn, IL) and timelapse images were analyzed and processed using Volocity software (Perkin-Elmer, Waltham, MA) as described previously[36].

References

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