Supplementary Figures, Legends, and Tables.

Figure S1

a) Genomic structure of the zebrafish wnt16 orthologue. a) Genomic structure of the zebrafish wnt16 locus and mRNA splice variants, with 5' and 3'-UTR (yellow) and open reading frames (ORFs; purple) found on four exons (top). Locations of the splice junctions blocked by W16MO1 and W16MO2 are indicated, as are locations of RT-PCR primers described in the text. Alternative splice acceptors are found 52 bps apart (blue box) in the second exon. Splicing to the 5'-acceptor yields two ORFs (middle, wnt16s): a highly truncated 5'-ORF that encodes a peptide of 40 amino acids, and a downstream ORF encoding a short-form protein of 239 amino acids, lacking a putative signal peptide, that exhibits no biological activity when injected (Fig. S2). Splicing to the more 3'-splice acceptor results in a message (bottom, wnt16l) with an ORF encoding a
predicted protein of 356 amino acids that has biological activity when injected (Fig. S3); high homology to mouse Wnt16, as well as human WNT16a and WNT16b (b); and a predicted signal peptide (boxed in turquoise). c) Phylogenetic cosegregation of zebrafish Wnt16 with mammalian Wnt16 proteins, as determined using PHYLIP software and bootstrap analysis (100 replicates) using the Neighbor-Joining distance method. d) Conservation of synteny between zebrafish wnt16 (top), human WNT16 (middle), and mouse Wnt16 (bottom). Schematic of relevant chromosomal regions with dashed lines linking homologous genes. Zebrafish si:ch211-152m4.1, human C7orf58, and mouse A430107O13Rik are homologous genes with different Ensembl annotations.

**Figure S2**

Supplementary Figure 2. Expression of zebrafish wnt16. Expression of wnt16 by WISH at the embryonic stages indicated (a-e, g-k, m-p), with, in some cases, secondary processing for myod (red; a, c, d, i, j) to identify the location of the posterior somite compartment, and individual panels of the vascular/haematopoietic marker scl (f, l) for comparison. Flat mount (a-c, e-f, i), close up lateral trunk (d, j) or head (p), lateral (g, k-n), and dorsal (h, o) views are shown with anterior to the left.
Supplementary Figure 3. Phenotypes caused by injection of \textit{wnt16} mRNA. A representative uninjected embryo (a) at 22 hpf (26-ss) compared to sibling embryos displaying a range of phenotypes (b-e) caused by injection of 70 pg \textit{wnt16l} isoform mRNA. A representative uninjected embryo at 23 hpf (f) compared to a representative embryo injected with 70 pg \textit{wnt16s} isoform mRNA (g).
Supplementary Figure 4. Effectiveness of wnt16 morpholinos. cDNA from embryos injected with W16MO1 (a) or W16MO2 (b) was subjected to RT-PCR analysis using RT1 (a) or RT3 (b) primers depicted in Fig. S1, and ef-1α as a control. For W16MO1 no higher molecular weight products were observed, indicating that message was degraded by nonsense-mediated decay. The intron-trapped product (indicated, b) produced by W16MO2 injection, was verified by sequencing and leads to message with an introduced stop codon after amino acid 31 (c). WISH demonstrates that the wnt16 transcript recognized by the wild-type probe is present at normal levels in uninjected embryos (278/278; d) and embryos injected with a control 5-base-mismatch morpholino (124/124; e), but not embryos injected with W16MO1 (181/215; f) or W16MO2 (130/147; g). d-g, 17 hpf (16-ss) embryos, dorsal views, with anterior to the left.
Supplementary Figure 5. Wnt16 control morpholino effects. Embryos injected with 5ng of a control morpholino with 5 bp mismatches compared to W16MO1 have unaffected Wnt16-regulated gene expression. Brightfield (a), runx1 (b), cmyb (c), rag1 (d), cd41:GFP (e), cmyb:GFP;kdrl:RFP (f), dlc (g), dl/dmyod (h), pax1 (i), foxc1a (j), foxc1b (k), twist1b (l). Red arrowheads mark dorsal aorta (b, c), or HSCs (e). Green arrowhead marks neural expression (b). Yellow arrows mark pronephric multiciliate cells (e). Blue arrows mark thymi (d). a, lateral; b, c, e, f, i-l close up lateral of trunk/aorta region; d, ventral; and g, h, flat mount. a-c, e-l anterior left; d, anterior up.
Supplementary Figure 6. Thymic epithelium is intact in W16MO animals. Expression of the lymphocyte marker *rag1* (a-b''), the thymic epithelium marker *foxn1* (c-d''), or both (e-f'') in uninjected (left panels) or W16MO-injected animals (right panels) as indicated. Right (a-f) and left (a''-f'') lateral views and ventral (a'-f') views are shown. Blue arrowheads indicate T-cells (a-a'', e-f'') and/or thymi (c-f'').
**Supplementary Figure 7.** Selected Notch ligands and receptors unaffected by reduction of Wnt16 activity.

Comparison of the expression of *notch1b* (a-b, e-f), *notch3* (c-d, g-h), *notch1a* (i-j), *notch2* (k-l), *jag2* (m-n) and *dll4* (o-p) at the times indicated, in uninjected or W16MO-injected embryos, as indicated above each column. a-d, i-j, m-p, lateral views. g-h, k-l flat mounts. All anterior to the left.
Supplementary Figure 8. Wnt16 is non-canonical. 6 hpf (shield stage; top two rows) or 17 hpf (16-ss; bottom row) embryos. Uninjected (left column), injected with 70 pg wnt3 mRNA (center column), or 70 pg wnt16 mRNA (right column) embryos, processed by WISH for the canonical target gene chd (top row), or GFP in Tg(TOP:GFP)w25 Wnt/β-catenin/Tcf-reporter animals (bottom two rows). a-f, animal views, dorsal to the right, where known. g-h, anterior left, dorsal up.

Supplementary Figure 9. Decreased Wnt16 activity does not alter β-catenin/Tcf-dependent Wnt pathway activity. Uninjected (a) or W16MO-injected (b) Tg(TOP:GFP)w25 animals processed by WISH for GFP show no difference in tissue-specific levels of β-catenin/Tcf-dependent Wnt signalling at 22 hpf.
Supplementary Figure 10. Wnt11 knock down does not affect HSCs. Wild-type (a) or cyclopic phenotype (b) confirms Wnt11 knockdown. Embryos processed for the HSC markers runx1 at 24 hpf (c-d) or cmyb at 36 hpf (e-f). Uninjected (left column) or W11MO-injected (right column). a-b, ventral head views. c-f close up lateral trunk views, anterior left.
Supplementary Figure 11. Decreased expression of a Notch reporter fluorophore in W16MO somites. GFP fluorescence in the somites (outlined with dashed white lines) and axial tissue of a transgenic animal carrying gfp under the control of a Notch-responsive promoter was examined by max-projection confocal imaging at 17.5 hpf in uninjected (a) or W16MO-injected (b) animals. Dorsal views, anterior left.

Supplementary Figure 12. Dorsal aorta expression of dlc in wnt16 morphants. Comparative expression of dlc in the dorsal aorta (red arrowhead) of uninjected (a) or W16MO-injected (b) embryos at 21 hpf (24-ss). Close up lateral views, anterior to the left.
Supplementary Figure 13. Dlc/Dld do not regulate wnt16. Expression of wnt16 at 17 hpf (16-ss) was compared to that of dlc (a) or dld (b) to examine overlap. Both overlapping and non-overlapping expression is observed. Wnt16 expression in uninjected (c), W16MO-injected (d), dlc mRNA-injected (e), dlc+dld mRNA-injected (f), bea homozygous mutants (g), and dldMO-injected animals (h). Only W16MO causes an alteration in wnt16. All lateral views, anterior left.
Supplementary Figure 14. Expression of Shh target genes in wnt16 morphants. Expression of the indicated Shh target genes was examined in uninjected or W16MO-injected animals as indicated above each column, at the time points noted. No significant alteration was observed. a-d close up lateral views. e-l lateral views. All anterior to the left.

|       | Uninj | W16MO | Uninj | W16MO |
|-------|-------|-------|-------|-------|
| a     | vegfaa| vegfaa| c     | prdm1a|
| b     | vegfaa| vegfaa| d     | prdm1a|
| e     | ptc1  | ptc1  | f     | ptc1  |
| i     | gli1  | gli1  | j     | gli1  |
| k     | nkl2.2a| nkl2.2a| l     | nkl2.2a|

22 hpf 22 hpf 23 hpf 23 hpf 22 hpf 22 hpf 22 hpf 22 hpf
Supplementary Figure 15. Wnt16 and Dlc/Dld are required for sclerotome patterning. Expression of the sclerotomal markers *pax1* (a-e), *foxc1a* (f-j), *foxc1b* (k-o), *twist1b* (p-t), and *twist2* (u-y), with *myod* (a-d) shown for reference in red. Uninjected, homozygous *bea*, wild-type injected with dldMO, homozygous *bea* injected with dldMO, or W16MO embryos are shown as indicated above each column. Yellow arrowheads identify a dorsomedial somitic sclerotomal domain, when present. Green arrowheads identify the hypochord, when present (u-y). Close up lateral views of the trunk region, anterior left, dorsal up.
Supplementary Movie 1. HSCs in uninjected cd41:GFP transgenic animals. Timelapse imaging of the trunk region, where GFP+ HSCs first appear near the dorsal aorta, in cd41:GFP transgenic animals visualized from approximately 50 hpf to approximately 75 hpf, at one frame every 3 mins. In the reference frames, red arrows indicate HSCs, and yellow arrows indicate multiciliate cells in the pronephros. Bright, rapidly circulating cells that begin appearing at about ~5 secs. (~53 hpf) are thrombocytes. Labelled reference frames have been added at 0 secs. (~50 hpf), 14 secs. (~60 hpf) and at 37 secs. (~75 hpf). Images were captured in parallel to the images presented in Movies S2-S4. Anterior to the left and dorsal up.

Supplementary Movie 2. HSCs are greatly reduced in W16MO-injected cd41:GFP transgenic animals. Timelapse images of W16MO-injected embryos, captured in parallel to the images presented in Movies S1, S3-S4, reveal vastly fewer HSCs, but normal multiciliate cells of the pronephros. Labelled as for Movie S1. Anterior to the left and dorsal up.

Supplementary Movie 3. Thymic immigration of lymphocyte precursors in cd41:GFP animals. Timelapse images of the head region of uninjected cd41:GFP transgenic animals captured in parallel to the images presented in Movies S1-S2, S4. The prospective thymus (50 hpf), thymic rudiment (60 hpf), and thymus (75 hpf) are identified with blue arrows in reference frames added at 0 secs., 14 secs., and 37 secs., respectively. Lymphocyte precursors retain the GFP label allowing visualization of their immigration. Anterior to the left and dorsal up.

Supplementary Movie 4. Greatly reduced thymic immigration of lymphocyte precursors in W16MO-injected cd41:GFP transgenic animals. Timelapse images of W16MO-injected embryos, captured in parallel to the images presented in Movies S1-S3, reveal vastly fewer lymphocyte precursors immigrating to the thymus. Labelled as for Movie S3. Anterior to the left and dorsal up.
### Table S1. Wnt16 mRNA overexpression effects.

| Morphology       | wild-type | dorsalized | A/P Defects\(^1\) | Other   |
|------------------|-----------|------------|---------------------|---------|
| uninjected       | 232/235   | 0/235      | 0/235               | 3/235   |
| 70pg wnt16s      | 34/34     | 0/34       | 0/34                | 0/34    |
| 70pg wnt16l      | 28/199    | 18/199     | 140/199             | 13/199  |

\(^1\)“A/P Defects” comprise anterior/posterior extension defects.

### Table S2. W16MO effects on HSC-associated genes.

| HSC Genes | treatment         | wild-type | weak | strong |
|-----------|-------------------|-----------|------|--------|
| runx1\(^1\) | Uninj             | 202/251   | 49/251| 0/251  |
|           | 5ng CoMO          | 8/13      | 5/13 | 0/13   |
|           | 5ng W16MO         | 39/249    | 16/249| 194/249|
| cmyb\(^1\)  | Uninj             | 96/109    | 13/109| 0/109  |
|           | 5ng CoMO          | 27/34     | 7/34 | 0/34   |
|           | 5ng W16MO         | 27/116    | 26/116| 63/116 |
| cd41:GFP   | Uninj             | 98/98     | 0/98 | 0/98   |
|           | 5ng CoMO          | 121/125   | 4/125| 0/125  |
|           | 5ng W16MO         | 52/145    | 9/145| 84/145 |
| rag1\(^2\)  | Uninj             | 39/40     | 1/40 | 0/40   |
|           | 5ng CoMO          | 12/12     | 0/12 | 0/12   |
|           | 5ng W16MO         | 2/58      | 15/58| 41/58  |

\(^1\)“Wild-type” denotes abundant positive cells in the aortic region. “Weak” denotes intermediate decrease. “Strong” denotes very few or no positive cells. 

\(^2\)“Wild-type” denotes abundant small round cells between the dorsal aorta and posterior cardinal vein. “Weak” denotes intermediate decrease. “Strong” denotes very few, or no positive cells.

\(^3\)“Wild-type” denotes strong bilateral thymic rag1\(^+\) cells. “Weak” denotes decreased or one-sided rag1\(^+\) cells. “Strong” denotes almost no, or no rag1\(^+\) cells.
### Table S3. W16MO effects on control genes.

| Unaffected Genes | treatment | wild-type | weak | strong |
|------------------|-----------|-----------|------|--------|
|                  |           |           |      |        |
| gata1            | Uninj     | 33/36     | 3/36 | 0/36   |
|                  | 5ng W16MO | 49/49     | 0/49 | 0/49   |
| myod             | Uninj     | 29/29     | 0/29 | 0/29   |
|                  | 5ng W16MO | 38/42     | 4/42 | 0/42   |
| tll1             | Uninj     | 49/49     | 0/49 | 0/49   |
|                  | 5ng W16MO | 37/55     | 12/55| 6/55   |
| cdh5             | Uninj     | 32/32     | 0/32 | 0/32   |
|                  | 5ng W16MO | 17/17     | 0/17 | 0/17   |
| flik1            | Uninj     | 34/34     | 0/34 | 0/34   |
|                  | 5ng W16MO | 31/31     | 0/31 | 0/31   |
| efnb2a           | Uninj     | 53/53     | 0/53 | 0/53   |
|                  | 5ng W16MO | 55/55     | 0/55 | 0/55   |
| col2a1a          | Uninj     | 85/90     | 5/90 | 0/90   |
|                  | 5ng W16MO | 72/79     | 7/79 | 0/79   |
| cdh17            | Uninj     | 30/30     | 0/30 | 0/30   |
|                  | 5ng W16MO | 45/45     | 0/45 | 0/45   |
| foxn1            | Uninj     | 14/14     | 0/14 | 0/14   |
|                  | 5ng W16MO | 0/9       | 0/9  | 0/9    |
| shha             | Uninj     | 20/20     | 0/20 | 0/20   |
|                  | 5ng W16MO | 20/20     | 0/20 | 0/20   |
| gfp              | Uninj     | 102/102   | 0/102| 0/102  |
|                  | 5ng CoMO  | 26/26     | 0/26 | 0/26   |
|                  | 5ng W16MO | 73/73     | 0/73 | 0/73   |

1Some effects in this group may reflect heterochronicity. 2WISH processing for GFP transcripts in TOP:GFP transgenic animals.

### Table S4. W11MO effects on HSC genes.

| Wnt11 Morpholino Phenotypes | treatment | wild-type | weak effect | strong effect |
|-----------------------------|-----------|-----------|-------------|---------------|
|                             |           |           |             |               |
| runx1                       | Uninj     | 19/20     | 1/20        | 0/20          |
|                             | W11MO     | 25/29     | 4/29        | 0/29          |
| cmyb                        | Uninj     | 20/20     | 0/20        | 0/20          |
|                             | W11MO     | 9/16      | 7/16        | 0/16          |
| cyclophia                   | Uninj     | 40/40     | 0/40        | 0/40          |
|                             | W11MO     | 0/45      | 0/45        | 45/45         |
Table S5. W16MO and CoMO effects on Notch pathway genes.

| Notch Pathway Genes | treatment | wild-type | weak | strong |
|---------------------|-----------|-----------|------|--------|
| **dlc (16-18hpf)**  | Uninj     | 51/51     | 0/51 | 0/51   |
|                     | 5ng CoMO  | 24/27     | 3/27 | 0/27   |
|                     | 5ng W16MO | 0/63      | 7/63 | 56/63  |
| **dlc (22-25hpf)**  | Uninj     | 113/114   | 1/114| 0/114  |
|                     | 5ng CoMO  | 64/67     | 3/67 | 0/67   |
|                     | 5ng W16MO | 134/174   | 30/174| 10/174 |
| **dll4**            | Uninj     | 76/76     | 0/76 | 0/76   |
|                     | 5ng CoMO  | 52/73     | 21/73| 0/73   |
|                     | 5ng W16MO | 20/120    | 47/120| 53/120 |
| **jag1b**           | Uninj     | 93/96     | 3/96 | 0/96   |
|                     | 5ng CoMO  | 30/36     | 6/36 | 0/36   |
|                     | 5ng W16MO | 19/81     | 56/81| 6/81   |
| **jag2**            | Uninj     | 28/28     | 0/28 | 0/28   |
|                     | 5ng CoMO  | 17/17     | 0/17 | 0/17   |
|                     | 5ng W16MO | 35/35     | 0/35 | 0/35   |
| **notch1a**         | Uninj     | 28/28     | 0/28 | 0/28   |
|                     | 5ng CoMO  | 18/18     | 0/18 | 0/18   |
|                     | 5ng W16MO | 34/34     | 0/34 | 0/34   |
| **notch1b**         | Uninj     | 145/145   | 0/145| 0/145  |
|                     | 5ng CoMO  | 79/80     | 1/80 | 0/80   |
|                     | 5ng W16MO | 134/191   | 41/191| 19/191 |
| **notch2**          | Uninj     | 41/41     | 0/41 | 0/41   |
|                     | 5ng CoMO  | 17/17     | 0/17 | 0/17   |
|                     | 5ng W16MO | 29/29     | 0/29 | 0/29   |
| **notch3**          | Uninj     | 150/154   | 4/154| 0/154  |
|                     | 5ng CoMO  | 65/69     | 4/69 | 0/69   |
|                     | 5ng W16MO | 155/207   | 39/207| 13/207 |
### Table S6. Notch path perturbation effects on HSC-associated genes.

| HSC Genes | wild-type | weak | strong |
|-----------|-----------|------|--------|
| runx1     |           |      |        |
| uninj     | 99/111    | 12/111 | 0/111 |
| bea       | 12/68     | 44/68 | 12/68 |
| dldMO2    | 0/22      | 12/22 | 10/22 |
| bea+dldMO2| 0/31      | 0/31  | 31/31 |
| cmyb      |           |      |        |
| uninj     | 62/67     | 5/67 | 0/67   |
| bea       | 11/60     | 32/60 | 16/60 |
| dldMO2    | 0/12      | 5/12 | 7/12   |
| bea+dldMO2| 0/18      | 0/18 | 18/18 |
| rag1      |           |      |        |
| uninj     | 41/41     | 0/41 | 0/41   |
| bea       | 28/35     | 7/35 | 0/35   |
| dldMO2    | 27/28     | 1/28 | 0/28   |
| bea+dldMO2| 0/9       | 0/9  | 9/9    |

### Table S7. W16MO effects on Shh pathway genes.

| Shh Target Genes | treatment   | wild-type | weak | strong |
|------------------|-------------|-----------|------|--------|
| vegfaa           | Uninj       | 32/32     | 0/32 | 0/32   |
|                  | 5ng W16MO   | 42/53     | 11/53 | 0/53   |
| prdm1a           | Uninj       | 74/74     | 0/74 | 0/74   |
|                  | 5ng W16MO   | 56/56     | 0/56 | 0/56   |
| ptc1             | Uninj       | 62/62     | 0/62 | 0/62   |
|                  | 5ng W16MO   | 53/57     | 4/57 | 0/57   |
| ptc2             | Uninj       | 33/33     | 0/33 | 0/33   |
|                  | 5ng W16MO   | 36/36     | 0/36 | 0/36   |
| gli1             | Uninj       | 33/33     | 0/33 | 0/33   |
|                  | 5ng W16MO   | 44/44     | 0/44 | 0/44   |
| ntx2.2a          | Uninj       | 39/39     | 0/39 | 0/39   |
|                  | 5ng W16MO   | 50/50     | 0/50 | 0/50   |

### Table S8. Notch rescue of W16MO HSC phenotype.

|                | wild-type | decreased | increased |
|----------------|-----------|-----------|-----------|
| uninj          | 27/33     | 6/33      | 0/33      |
| uninj NICD+    | 2/11      | 0/11      | 9/11      |
| W16MO NICD- 14h hs | 9/73     | 63/73    | 2/73      |
| W16MO NICD+ 14h hs | 3/13     | 1/13     | 9/13      |
| W16MO NICD- 16h hs | 7/33     | 26/33    | 0/33      |
| W16MO NICD+ 16h hs | 4/14     | 10/14    | 0/14      |
| Sclerotome Genes | treatment       | wild-type  | weak   | strong  |
|------------------|-----------------|------------|--------|---------|
| **pax1**         | Uninj           | 160/165    | 3/165  | 2/165   |
|                  | 5ng CoMO        | 27/27      | 0/27   | 0/27    |
|                  | 5ng W16MO       | 4/155      | 39/155 | 117/155 |
|                  | bea             | 13/67      | 50/67  | 4/57    |
|                  | 7ng dldMO       | 5/48       | 43/48  | 0/48    |
|                  | bea+dldMO       | 0/52       | 10/52  | 42/52   |
| **foxc1a**       | Uninj           | 127/127    | 0/127  | 0/127   |
|                  | 5ng CoMO        | 14/14      | 0/14   | 0/14    |
|                  | 5ng W16MO       | 0/73       | 0/73   | 73/73   |
|                  | bea             | 0/68       | 68/68  | 0/68    |
|                  | 7ng dldMO       | 13/68      | 53/68  | 2/68    |
|                  | bea+dldMO       | 0/52       | 52/52  | 52/52   |
| **foxc1b**       | Uninj           | 92/92      | 2/94   | 0/94    |
|                  | 5ng CoMO        | 18/18      | 0/18   | 0/18    |
|                  | 5ng W16MO       | 0/91       | 0/91   | 91/91   |
|                  | bea             | 0/32       | 32/32  | 0/32    |
|                  | 7ng dldMO       | 6/39       | 33/39  | 0/39    |
|                  | bea+dldMO       | 0/34       | 2/34   | 32/34   |
| **twist1b**      | Uninj           | 55/55      | 0/55   | 0/55    |
|                  | 5ng CoMO        | 19/19      | 0/19   | 0/19    |
|                  | 5ng W16MO       | 0/41       | 8/41   | 33/41   |
|                  | bea             | 0/16       | 16/16  | 0/16    |
|                  | 7ng dldMO       | 6/14       | 8/14   | 0/14    |
|                  | bea+dldMO       | 0/37       | 0/37   | 37/37   |
| **twist2**       | Uninj           | 24/24      | 0/24   | 0/24    |
|                  | 5ng W16MO       | 0/23       | 5/23   | 18/23   |
|                  | bea             | 0/18       | 0/18   | 18/18   |
|                  | 7ng dldMO       | 3/22       | 5/22   | 14/22   |
|                  | bea+dldMO       | 0/17       | 0/17   | 17/17   |