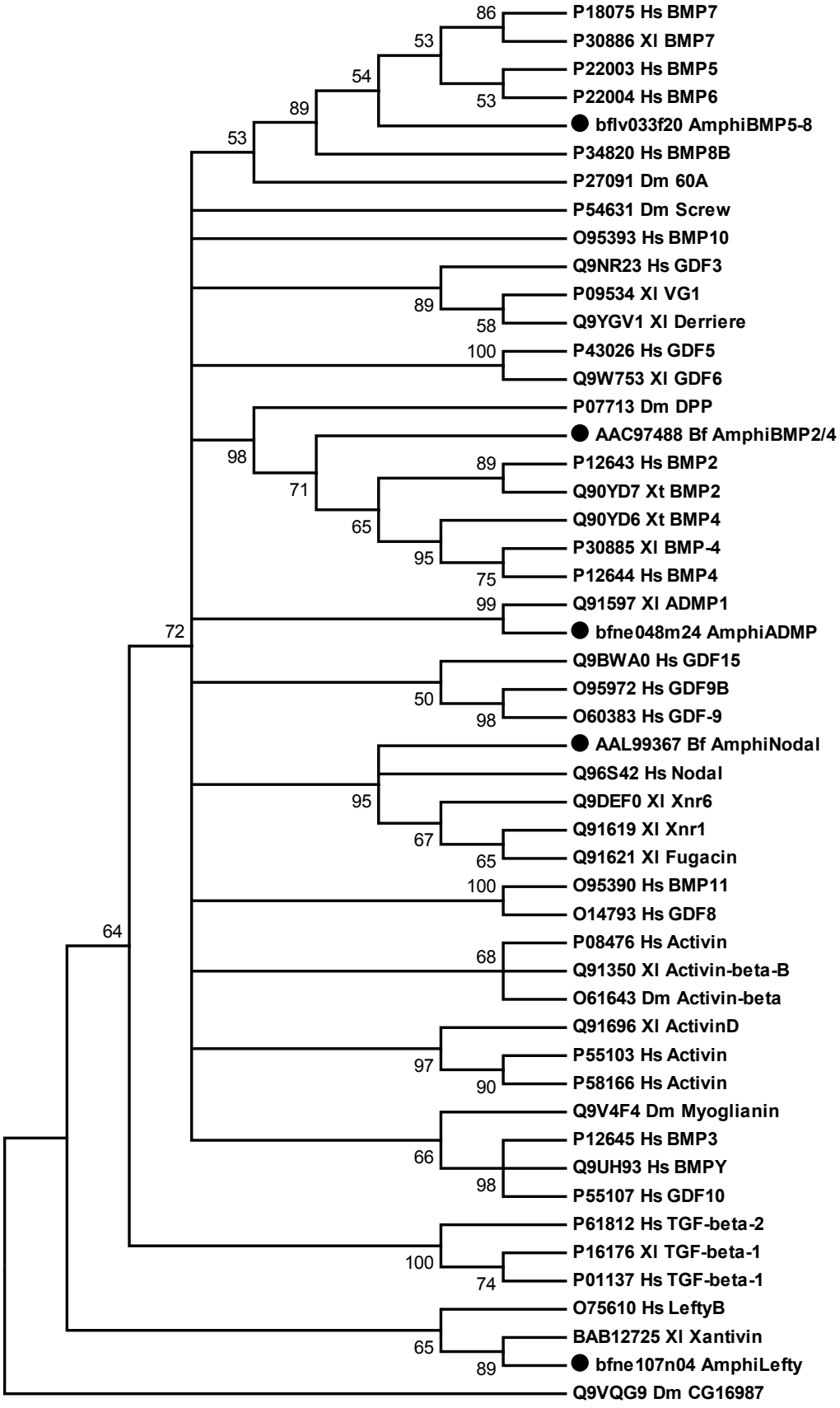
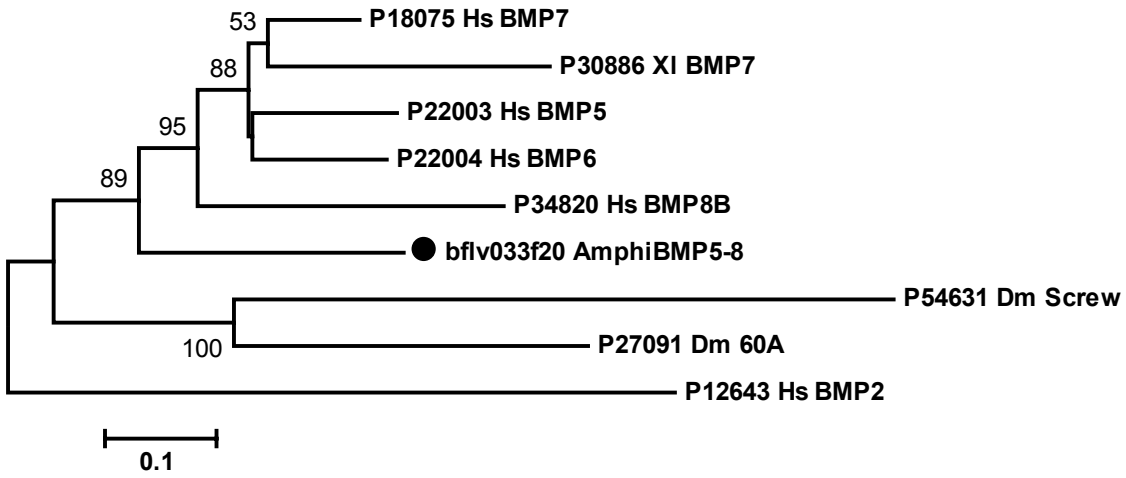
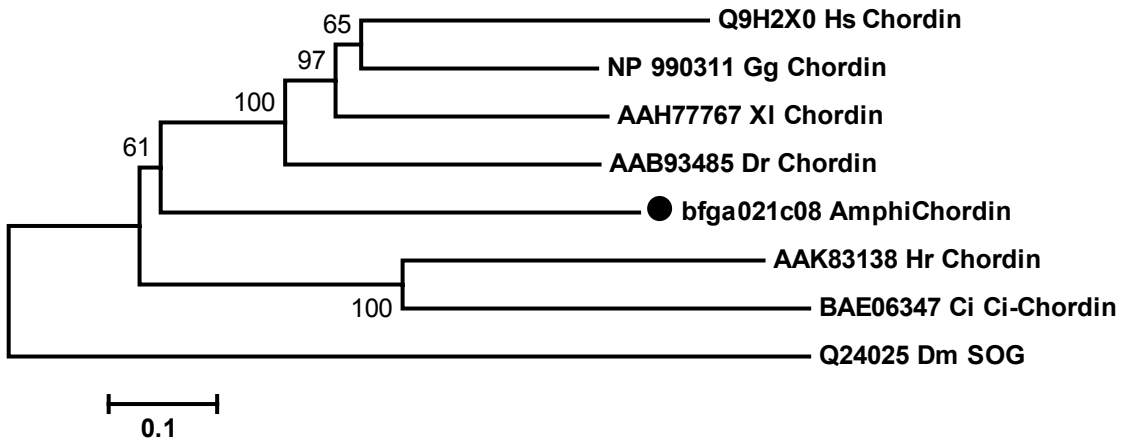
SUPPLEMENTARY INFORMATION



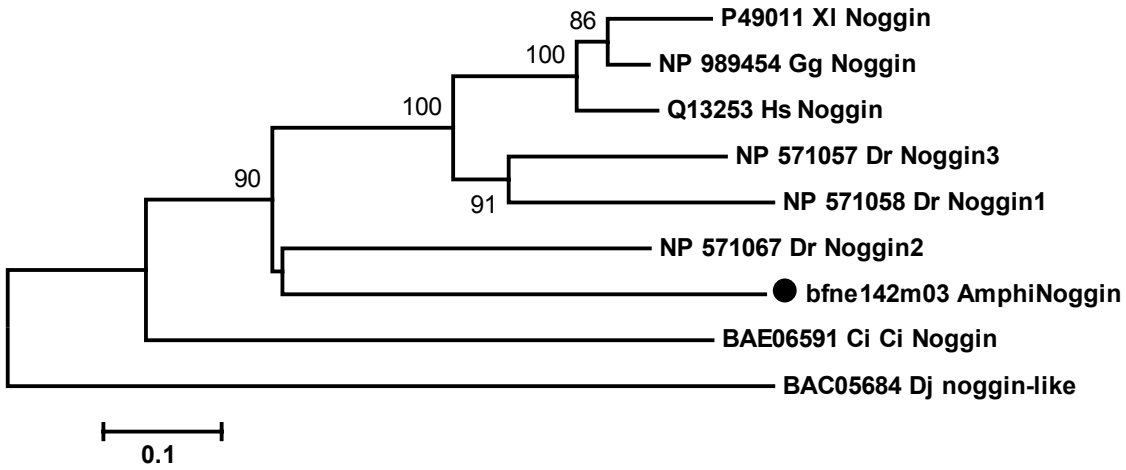
Supplementary Figure 1 Neighbor-joining bootstrap consensus tree reconstructed with the MEGA program for transforming growth factor β (TGFβ) proteins. Amphioxus proteins are shown by large black dots. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. Branches with values < 50% are not resolved. The tree was constructed with the C-terminal conserved mature region. The constructed tree shows that AmphiBMP2/4 is in the BMP2/4 (Dpp) subfamily (bootstrap value of 98%), AmphiADMP is in the ADMP subfamily (99%), AmphiNodal is in the Nodal subfamily (95%), and AmphiLefty is in the Lefty subfamily (65%). To examine phylogenetic relationships between the amphioxus BMP5-8 protein and other BMP5-8 proteins with higher resolution, phylogenetic trees were constructed using only BMP5-8 proteins plus outgroups with a longer alignment (Supplementary Figure 2).



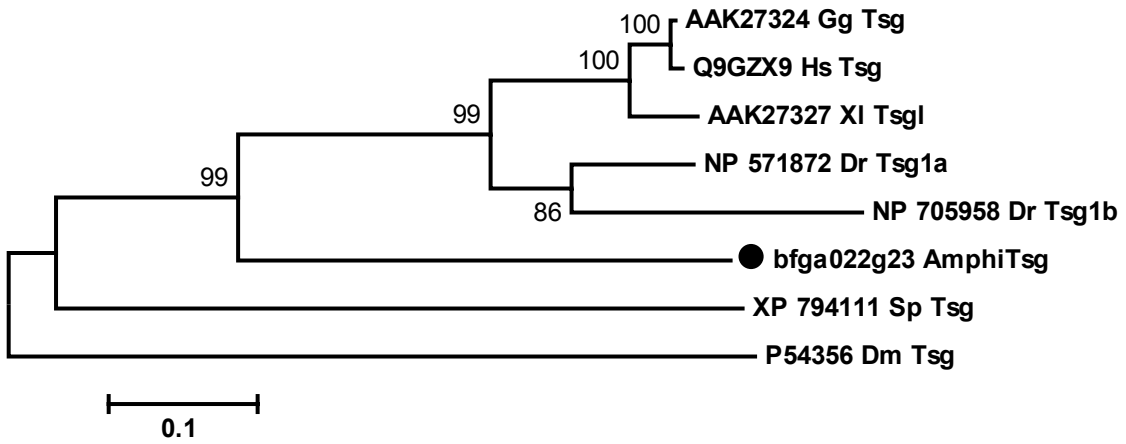
Supplementary Figure 2 Phylogenetic tree of BMP5-8 proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus BMP5-8 is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. *Homo sapiens* BMP2 protein was used as an outgroup. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.1 substitutions per position.



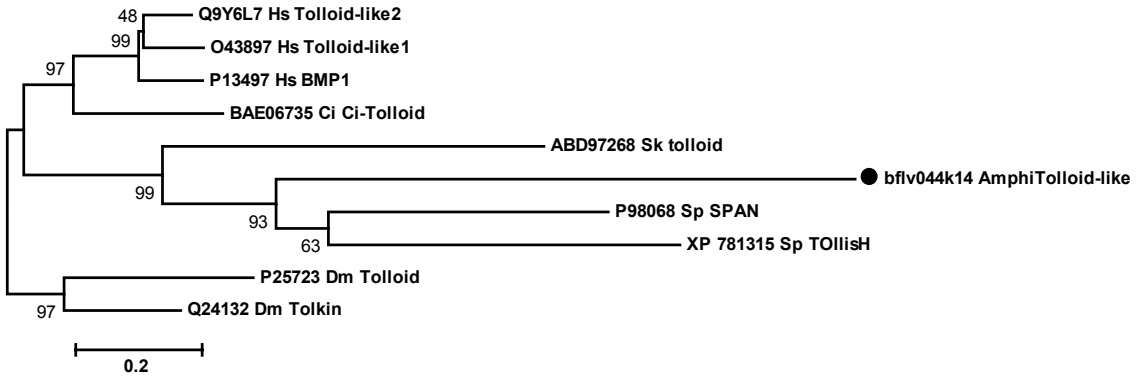
Supplementary Figure 3 Phylogenetic tree of Chordin proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Chordin is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. An unrooted tree is shown as a rooted tree for simplicity. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.1 substitutions per position.



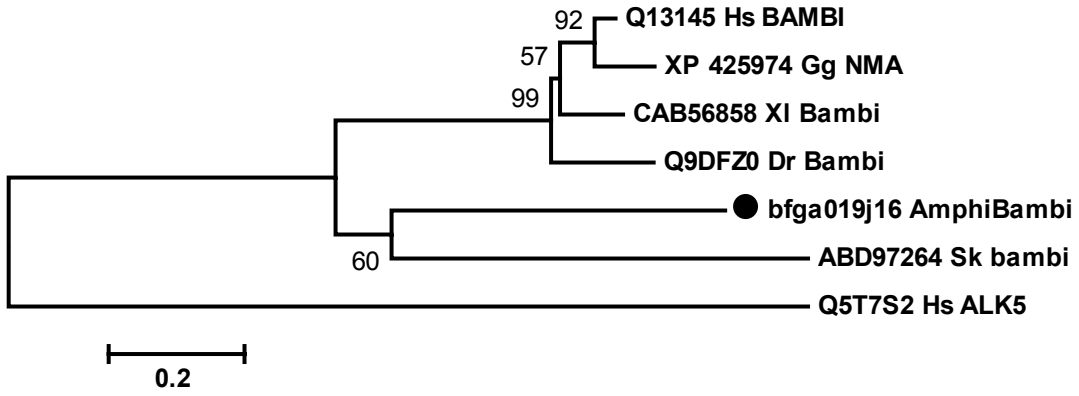
Supplementary Figure 4 Phylogenetic tree of Noggin proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Noggin is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. Planarian *Dugesia japonica* noggin was used as an outgroup. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.1 substitutions per position.



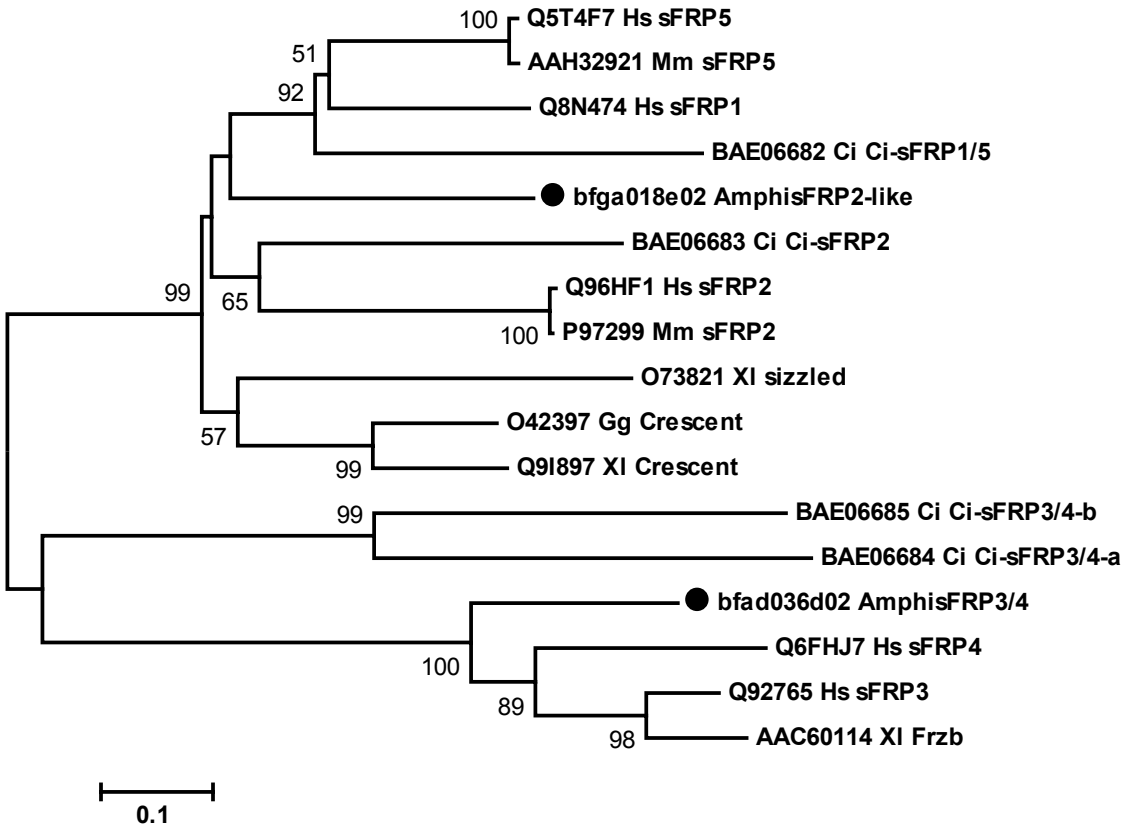
Supplementary Figure 5 Phylogenetic tree of Tsg proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Tsg is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. An unrooted tree is shown as a rooted tree for simplicity. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.1 substitutions per position.



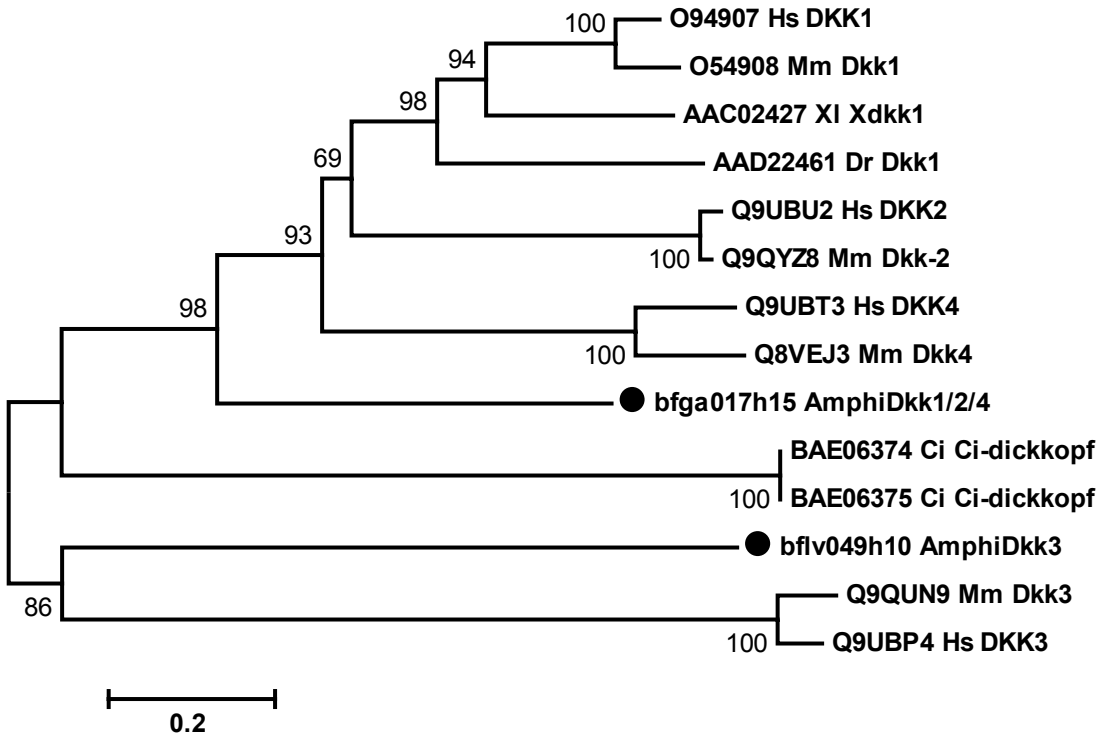
Supplementary Figure 6 Phylogenetic tree of Tolloid/BMP1 proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Tolloid-like is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. *Drosophila melanogaster* Tolloid and Tokin were used as outgroups. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.2 substitutions per position.



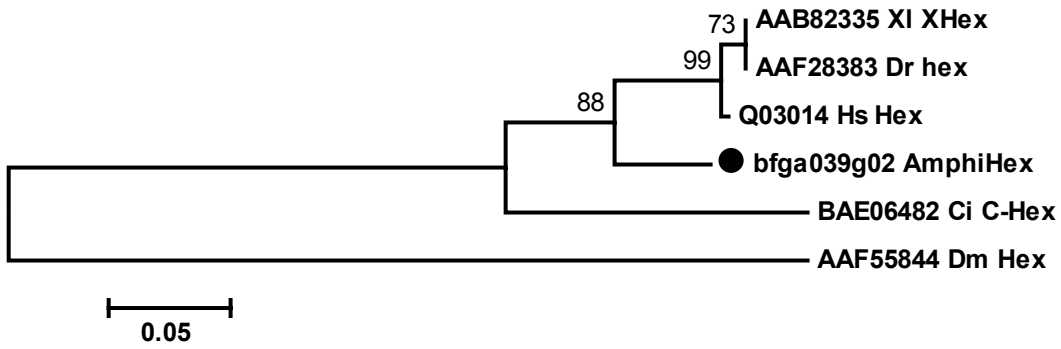
Supplementary Figure 7 Phylogenetic tree of BAMBI proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Bambi is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. *Homo sapien* TGFβ receptor I (ALK5) was used as an outgroup. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.2 substitutions per position.



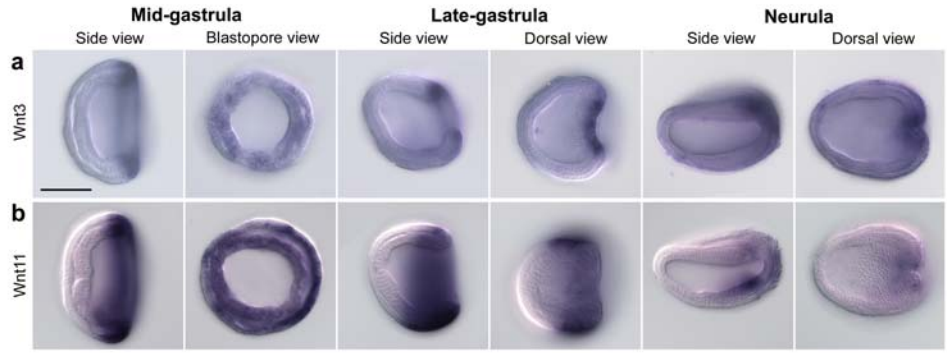
Supplementary Figure 8 Phylogenetic tree of secreted frizzled related proteins (sFRP) proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus proteins are shown by large black dots. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. An unrooted tree is shown as a rooted tree for simplicity. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.1 substitutions per position. In the present study we identified two sFRP genes in amphioxus, and the constructed tree shows that one of them, AmphisFRP3/4, is in the group including vertebrate sFRP3/Frzb and sFRP4. However, the present analysis failed to resolve the phylogenetic relationship between the remaining amphioxus sFRP and other known sFRP subfamilies. We designated this protein as a putative AmphisFRP2-like because its best-hit protein in the human proteome database was the sFRP2 protein.



Supplementary Figure 9 Phylogenetic tree of Dkk proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus proteins are shown by large black dots. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. An unrooted tree is shown as a rooted tree for simplicity. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.2 substitutions per position.



Supplementary Figure 10 Phylogenetic tree of Hex proteins, reconstructed with the MEGA program using the neighbor-joining method with default settings. Amphioxus Hex is shown by a large black dot. The values beside the branches represent the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. An unrooted tree is shown as a rooted tree for simplicity. Protein names are shown as explained in the Supplementary Methods. The scale bar indicates an evolutionary distance of 0.05 substitutions per position.



Supplementary Figure 11 Expression of amphioxus *Wnt3* and *Wnt11* during embryogenesis. For side and dorsal views of whole mounts, anterior is toward the left; for blastopore views, dorsal is toward the top. **a**, *Wnt3* is expressed restricted to a rim around the blastopore. **b**, *Wnt11* is also expressed around the blastopore, but the expression domain is broader than that of *Wnt3*. Scale bar, 50 μm.

Supplementary Table 1. Organizer genes in the amphioxus ESTs: representative clones

Gene name	Clone ID	No. of clones	The best hit protein in the human proteome	E value
BMP family members, BMP antagonists, and their modulators				
AmphiBMP2/4	bfga034p10	27	P12643 BMP-2	1.00E-113
BMP5-8	bflv033f20	7	P18075 BMP-7	1.00E-100
ADMP	bfne048m24	3	P12643 BMP-2	2.00E-42
Chordin	bfga021c08	11	Q9H2X0 Chordin	1.00E-175
Noggin	bfne142m03	1	Q13253 Noggin	3.00E-45
Tsg	bfga022g23	13	Q9GZX9 Twisted gastrulation	2.00E-57
Tolloid-like	bflv044k14	10	O43897 Tolloid-like protein 1	4.00E-33
BAMBI	bfga019j16	7	Q13145 BAMBI	7.00E-34
Nodal and its antagonist				
AmphiNodal	bfne084e19	16	Q96S42 Nodal	9.00E-35
Lefty	bfne107n04	3	O75610 Lefty-B	1.00E-30
Wnts and Wnt antagonists				
AmphiWnt3	bflv058m15	6	P56703 Wnt-3	1.00E-142
AmphiWnt8	bfne137b06	8	Q93098 Wnt-8b	1.00E-103
AmphiWnt11	bfga010i23	1	O96014 Wnt-11	1.00E-105
sFRP3/4	bfad036d02	2	Q92765 Secreted frizzled-related protein 3	1.00E-74
sFRP2-like	bfga018e02	21	Q96HF1 Secreted frizzled-related protein 2	2.00E-51
Dkk1/2/4	bfga017h15	9	Q9UBU2 Dickkopf related protein-2	2.00E-26
Dkk3	bflv049h10	2	Q9UBP4 Dickkopf related protein-3	2.00E-10
Transcription factors involved in organizer development				
Goosecoid	bfga017a22	19	P56915 Goosecoid	3.00E-18
AmphiVent	bfga019g16	21	P52954 LBX1	1.00E-14
AmphiEvxA	bfne099k15	8	Q03828 EVX-2	5.00E-49
Hex	bfga039g02	19	Q03014 Homeobox protein PRH (HEX)	5.00E-56

Supplementary methods

Phylogenetic analysis. The sequences were aligned using the Clustal X program[40], and the alignment was checked manually. After removing gaps, the verified alignments were used to construct phylogenetic trees with MEGA program version 2.1[41] based on the Neighbor-joining method. Bootstrap support values were calculated by 1000 pseudoreplications. The sequences used are designated by the accession number, abbreviation of the species, and gene name. For example, human BMP2 (accession number: P12643) is represented as "P12643 Hs BMP2". Species name abbreviations: Bf for *Branchiostoma floridae*, Ci for *Ciona intestinalis*, Dj for *Dugesia japonica*, Dm for *Drosophila melanogaster*, Dr for *Danio rerio*, Gg for *Gallus gallus*, Hr for *Halocynthia roretzi*, Hs for *Homo sapiens*, Mm for *Mus musculus*, Sk for *Saccoglossus kowalevskii*, Sp for *Strongylocentrotus purpuratus*, and Xl for *Xenopus laevis*. Molecular phylogenetic analysis of amphioxus Wnts, Goosecoid, Vent, and Evx have been published previously[17,20, 21, 42, 43].

In situ hybridization. Except for the genes mentioned below, we used a primer matching with the vector sequence adjacent to the 3'end of the cDNA insert with a T7 promoter sequence added to its 5'end as a reverse primer (pDONR222-T7-Reverse, 5'-TAATACGACTCACTATAGGGAGGGGATATCAGCTGGATG-3'), and a primer matching with the vector sequence adjacent to the 5'end of the cDNA insert with a SP6 promoter sequence added as a forward primer (pDONR222-SP6-Forward, 5'-ATTTAGGTGACACTATAGAAGACGGCCAGTCTTAAGCTC-3') to PCR amplify the full-length cDNA for antisense riboprobe synthesis. For *Tsg*, *sFRP3/4*, and *Lefty*, only the 5' half

cDNA fragments were amplified by pDONR222-SP6-Forward primer and a gene specific primer with a T7 promoter sequence added for riboprobe synthesis. The sequences of the gene specific primers are: *Tsg*, 5'- TAATACGACTCACTATAGGGCGCTCATACAGTTCGAGCAC-3'; *sFRP3/4*, 5'- TAATACGACTCACTATAGGGCTCACCCTTCGCGATCAAC-3'; *Lefty*, 5'- TAATACGACTCACTATAGGGGCTCCAGGATGAACTGCTC- 3'. The PCR protocol was: 2 min 94°C; 5 cycles: 30s 94°C, 30s 45°C, 3 min 68°C; 25 cycles: 30s 94°C, 30s 58°C, 3 min 68°C; 1 cycle 7min 68°C. The riboprobe for amphioxus *Nodal* gene was described previously[14]. DIG-labeled or fluorescein-labeled antisense ribobrobes were synthesized by T7 RNA polymerase. The procedure of in situ hybridization for amphioxus embryos was performed as described[14]. Detection was with alkaline phosphatase conjugated anti-DIG or anti-fluorecein antibodies. Alkaline phosphatase reaction products were visualized with NBT-BCIP (purple color for DIG-labeled probes) or BCIP only (cyan color for fluorescein-labeled probes).

Equipment and settings. After in situ hybridization, photographs were taken under DIC optics with a Zeiss Axiocam mounted on a Zeiss Axioskop 2 microscope. Lettering and scale bars were added with Adobe Photoshop version 7.0. Whole images were minimally and uniformly adjusted for brightness and contrast. The line diagram in Figure 5 was constructed with Deneba Canvas version 9.0 and converted to jpg format.

Supplementary Notes

References

40. Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G. & Gibson, T. J. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**, 403-405 (1998).

41. Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**, 1244-1245 (2001).

42. Schubert, M., Holland, L. Z., Holland, N. D., & Jacobs, D. K. A phylogenetic tree of the Wnt genes based on all available full-length sequences, including five from the cephalochordate amphioxus. *Mol. Biol. Evol.* **17**, 1896-1903 (2000).

43. Schubert, M., Holland, L. Z., Stokes, M. D. & Holland, N. D. Three amphioxus Wnt genes (*AmphiWnt3*, *AmphiWnt5*, and *AmphiWnt6*) associated with the tail bud: the evolution of somitogenesis in chordates. *Dev. Biol.* **240**, 262-273 (2001).