Supporting Information

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SI Materials and Methods

Cloning

Wnt5. Two Wnt5-related fragments (accession nos. CN631317 and CN630976) were detected in Hydra EST collections, and their sequence information was used for further cloning. To characterize the 5′ end of the *hvwnt5* mRNA, repeated 5′ RACE was done by using the SMART RACE cDNA Amplification Kit (Clontech). The complete *hvwnt5* coding region was cloned by PCR using *H. vulgaris* cDNA. RACE- Primers: Wnt5-R5, CACAACACAAACGACCGCATTC; Wnt5-R4, CGTCTTTGATGCATTCTCGACC; Wnt5-R8, CCCCATGACAACGGCATTGGATC; Wnt5-R7, CTGCCCATCTCCCTGCTTCGT; Wnt5-R6, GTGAAGGTTCATTAACACTCTTCC; Wnt5-R12, GCTGTTTCACGAGTTCCAAGTGG; Wnt5-R11, CGAGTTCCAAGTGGCATGTAAGG. PCR-Primers, Wnt5-ESTeF, GGATATCAGCTAGAGCAACTCC; Wnt5-ESTeR, CGCAAACATGGGTTATTTCG; Wnt5-mRNAF3, GAAGATGGCAAATCACCAAAG; Wnt5-mRNAF4, AAAAAGAAGATGGCAAATCA; HyWnt5-R3, GACCTTCCGTTCCAAATGAACC; HyWnt5-R4, CGTCTTTGATGCATTCTCGACC.

Wnt8. Two Wnt8/11-related fragments (accession nos. CN552348 and CN552013) were detected in Hydra EST collections, and their sequence information was used for further cloning. To characterize the 5′ end of the *hvwnt8* mRNA, 5′ RACE was done by using the 5′ RACE Gene Racer Kit (Invitrogene). To clone the corresponding 3′ end, 3′ RACE was done by using the classic Frohmann method. RACE-Primers: Wnt11–4, GAAGAAAGTGAAGATTTCCAGC; Wnt11–3, CTTGAACAATAGCCGGAGAGTCC; Wnt11-mRNAF3, CAATGCAAAATGGATAGA; Wnt11-mRNAF4, AAAATCAATGCAAAATGGAT; Wnt11–3, CTTGAACAATAGCCGGAGAGTCC; Wnt11-ESTaF, TATGCAGTTCAATGGCGGCAC; Wnt11-ESTaR, TGCTCTGTGTACTGGGATCCG; Wnt11-ESTeR2, GATTGCACGTTTACTCGACAC.

Frizzled2. A partial cDNA clone (accession no. DT614590) encoding a Frizzled protein fragment was identified in the *Hydra magnipapillata* EST collection in the NCBI database. The entire ORF of the corresponding transcript was retrieved from contig 36976 of the *H. magnipapillata* genome assembly. In BLAST searches, the predicted amino acid sequence showed highest similarity to members of the Frizzled 2/5/8 subfamily. Using sequnce-specific primers (Fz2fwd, CGTTATAATCCATCTGCTCTG; Fz2rev, GCTAGATATTGTACCGTTGGTCC), a DNA fragment of 1,023-bp length was amplified by PCR from a random-prime *Hydra* cDNA, cloned in *Escherichia coli*, and used to produce a DIG-labeled RNA probe for in situ hybridization.

Rho-associated kinase (Rok). HvRok was identified in a PCR approach by using degenerated primers against the highly conserved N-terminal part. The complete ORF of the *hvrok* mRNA was then characterized by using 5′ and 3′ RACE experiments. Degenerated PCR primers: Rok2, GCNTTYTTYTGGGARGA; Rok6, CANGTNCCRAARTCNGC. RACE primers, Rok7, AATGAATCGCCATAATCCATTCAG; Rok9, AATGAATCGCCATAATCCATTCAG; Rok11, TCATAAGTATGGCTATGTTCACAG; Rok12, GACATAAAACCAGACAACATGCTG; Rok14, GGAAATCATTTGCCATTCATTGG; Rok15, GGAGGAAATCAGGGAACATCTG; Rok17, AAAGTGATGCCAATGCCATGC; Rok19, GCCTTTGGAATCAAGCTCCATC; Rok20, GAAACAATATGTAGTTGTAAGCCGG; Rok21, GCAACTAATACACCTTCTATGATC; Rok23, GCTGCTGAATGTCGAAGATGTCG; Rok25, AACTGTTGATCTTGCTACTGCC.

Strabismus (Stbm). HvStbm was identified in a PCR approach by using degenerated primers against conserved motifs in the middle part of the protein. The complete ORF of the *hvrok* mRNA was then characterized by using 5′ and 3′ RACE experiments. Degenerated PCR primers: Stbm1, GCNGCNGCNMGNMGNMG; Stbm2, ACNANNCGNCMNCGGTA; Stbm3, GARGANGCNTTYWSNCA; Stbm4, ACNSWYTTNCGNAGRAG. RACE primers, Stbm5, AGTGCTTTCTTGAACCATCTAA; Stbm6, TCTCTAACACATTTACAGCAGC; Stbm7, GAAGTAGGACGTGGAAGGTATGG.

Carbon Labeling and Morphometric Measurements. Clusters of 20 to 30 ectodermal epithelial cells close to the base of early evaginating buds and tentacles were vitally labeled by injecting ink (Pelikan) into the interstitial spaces of the ectodermall cell layer. Epithelial cells incorporate and permanently store ink (carbon) particles within vacuoles; 2 h after labeling (t_0), and after 3 days (t_3), the carbon-labeled cell clusters were analyzed under phase contrast optics. Their maximal lengths were determined along the major body axis of the parental polyp (oral-aboral) and along the axis of the evaginated bud or tentacle (distal-proximal). The length ratio of individual clusters between t_3 and t_0 was calculated for each individual *Hydra* and plotted as x-fold length. Measurements were done in living *Hydra* relaxed with 2% urethane in hydra medium.

Inhibitor Treatments. Treatment with SP600125 (A.G. Scientific) and ZTM000990 (Novartis Pharma AG) started with incubation of experimental animals at a density of 1 polyp per milliliter in inhibitor(s) diluted in 5% DMSO/hydra medium for 30 min on ice. Then, after 3 short washes in 0.1% DMSO/hydra medium, animals were transferred to inhibitor(s) diluted in 0.1% DMSO/hydra medium in the dark. Solutions were replaced daily during long-term treatments. Treatment with 5 μM alsterpaullone (A. G. Scientific) in 0.1% DMSO/hydra medium was done in the dark for 24 h. Thereafter, polyps were cultured further in hydra medium. In cotreatment experiments, animals were incubated in 5 μM alsterpaullone/25 μM SP600125 or 5 μM alsterpaullone/25 μM ZTM000990 diluted in 0.1% DMSO/hydra medium in the dark for 24 h. Phenotypes of treated polyps were analyzed 60 h after the onset of treatment. Control animals were carried through the experiments with the corresponding treatment schemes using DMSO only.

Kinase Assays and Phospho-Specific Antibodies. Polyps treated with SP600125 for 24 h were lysed in NuPAGE LDS sample buffer (Invitrogen) containing 1:100 phosphatase inhibitor (Sigma-Aldrich); c-Jun phosphorylating activity of the lysates was monitored by using a SAPK/JNK assay kit (9810, Cell Signaling Technology). In the same experiment, the supernatant of the immunoprecipitate was used to detect *Hydra* phospho-ERK proteins by using phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody (4370, Cell Signaling Technology). Kinase assays were performed according to the manufacturer's guidelines. ATF-2 phosphorylating activity of SP600125-treated tissue lysates was monitored by using a p38 MAP kinase assay kit (9820, Cell Signaling Technology). *Hydra* phospho-JNK levels were determined by using phospho-SAPK/JNK (Thr-183/Tyr-185) antibody (9251, Cell Signaling Technology). Anti-actin antibody (A2066, Sigma-Aldrich) was used to evaluate protein loading in all experiments. Proteins were separated by SDS/PAGE, blotted, and ECL detected by using standard protocols.

Immunoprecipitation of *Hydra* β-Catenin and Tcf. *Hydra β-catenin* complete cDNA was cloned into a pET15b bacterial expression vector (Novagen) using BamHI restriction sites and expressed in *E. coli* BL21 (DE3) cells. Histidine-tagged β-Catenin was purified by using a Ni-NTA column. *Hydra tcf* complete cDNA was clonded into a pGEX6p3 vector (Amersham) by using BamHI and NotI restriction sites and expressed in *E. coli* BL21 (DE3) cells. Purification of Tcf-GST was performed according to the manufacturer's instructions (Amersham); 1 μg of purified recombinant *Hydra* β-Catenin was bound to Ni-NTA agarose in PBS and incubated with an equimolar concentration of *Hydra* Tcf-GST and different concentrations of ZTM000990 for 4 h at 4 °C by shaking. Ni-NTA agarose beads were washed 3× with PBS/0.01% Tween20, and bound β-Catenin was released from the Ni-NTA beads by incubation in PBS containing 250 mM imidazole. Samples were analyzed for Tcf-GST by Western blotting with an anti-GST antibody at 1:2,000. In control experiments, Tcf-GST was incubated with Ni-NTA Sepharose without β-Catenin or only GST instead of Tcf-GST was used as a ligand. Also, ZTM000990 did not interfere with the binding of His-tagged *Hydra* β-Catenin to Ni-NTA beads even at concentrations of up to 250 μM.

RT-PCR. We treated 15 polyps of *H. magnipapillata* strain 105 with 25 μM ZTM000990, 5 μM alsterpaullone, or cotreated with both inhibitors. Polyps were lysed and total RNA was prepared by using an RNeasy kit (Qiagen), together with on-column DNA digest (DNase set, QIAGEN) according to manufacturer's instruction; ≈1 μg of total RNA of each sample was reversely transcribed into cDNA by using random primer. Gene expression was examined under the following conditions: 1.5 μL cDNA in 30 μL reaction scale, 200 μM dNTPs, 250 μM oligonucleotides, 3 mM $MgCl_2$, 0.25 μL Taq polymerase (Euroclone). Program: 94 °C 8 min; 94 °C 30 sec (denaturation), 55 °C 30 sec (annealing), 68 °C 40 sec (extension); cycles: 32 (*hytcf*), 32 (*hvwnt5*), 34 (*hvwnt8*), 28 (*Hychdl*), and 24 (*ef1α*). Primers: *hytcf*, 5′-TTATACCCGGGTTGGAGTGC and 5′-TCGAAGCGCACCAGAAGAGTAACAT; *hvwnt5*, 5′-ATGTTAACGAATTTCTTGAGTGGC and 5′-AGAGTTTAAAGATGTTGGATATCAGCT; *hvwnt8*, 5′-CTCTCCGGCTATTGTTCAAG and 5′-TTTCAATCGTCTTTACAAACGAATGTTTTC; *Hychdl*, 5′-ACCTGTCCTCCCTGCAATGAAGAAT and 5′-ACTGCGCAGTTCACATATGT; *ef1α*, 5′-GTTGGTCGTGTTGAAACTGG and 5′-TCCAGCAGCAACACCTGCTT. Primer pairs included introns in the corresponding genomic sequences to exclude contamination of the results by nuclear DNA.

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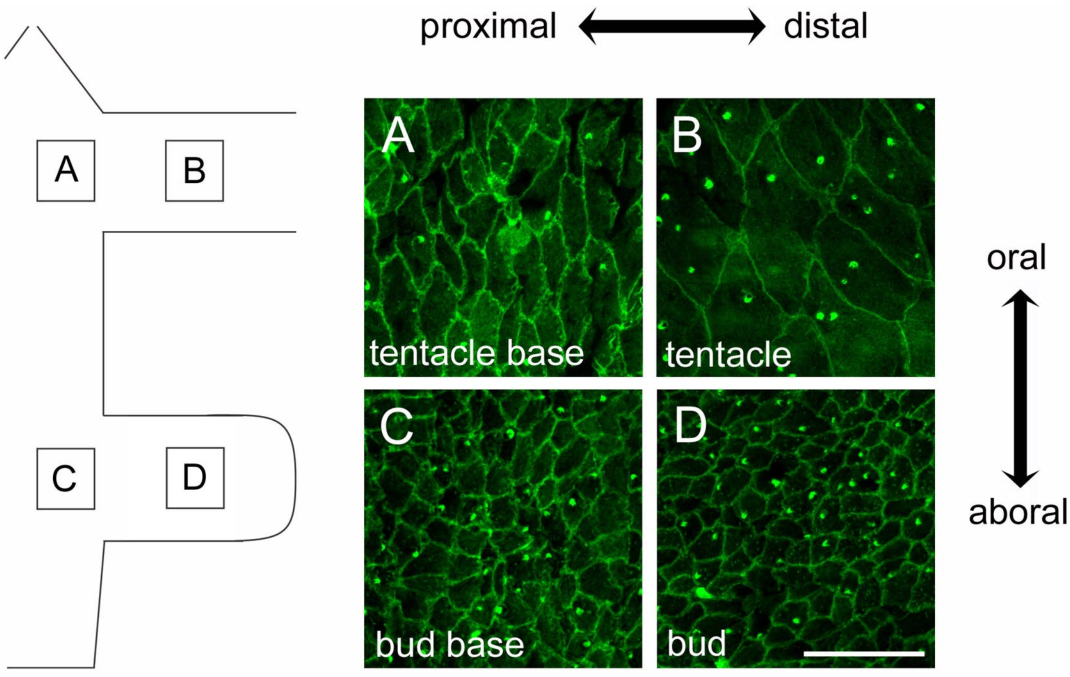

Apical cell diameters of ectodermal epithelial cells

	tentacle base	tentacle	bud base	bud
oral-aboral	27.9±3.9	43.9±10.4	16.6±2.7	13.6±2.4
distal-proximal	14.6±2.0	29.6±2.8	16.7±3.8	14.6±2.4

Fig. S1. Apical cell diameters of ectodermal epithelial cells in evaginated and unevaginated tissues. Polyps with 1 stage 5 bud were fixed and stained with fluorescent phalloidin according to the protocol described in the main article. Views onto the plane of the epithelium at the level of apical cell contact sites show a concentration of actin filaments. By using these preparations, planar diameters of ectodermal epithelial cells were measured at positions A–D as indicated in the representation. Maximal diameters of individual cells were determined along the oral-aboral and distal-proximal axes. Each value represents the mean ± SD of 100 epithelial cells from 5 different specimens.

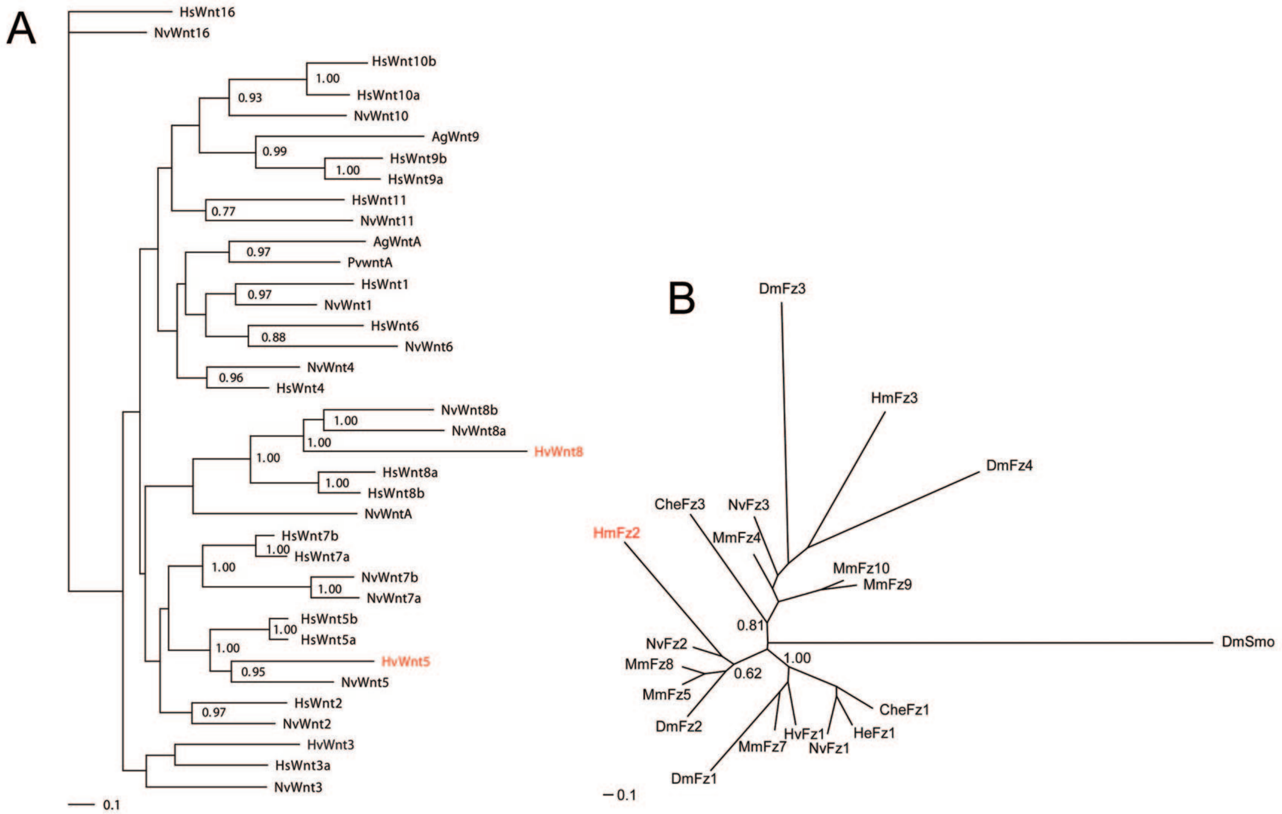

Fig. S2. Phylogenetic trees of Wnt and Frizzled subfamilies. (*a* and *b*) Maximum likelihood analysis with IQPNNI 2.2 (1) reveals a clustering of the predicted *Hydra* Wnt5 and Wnt8 amino acid sequences within the corresponding Wnt subfamilies supported by high support values from Bayesian analysis (*a*). A corresponding analysis using full-length Frizzled amino acid sequences places *Hydra* Frizzled2 at the basis of a Frizzled2/5/8 subfamily (*b*). *Drosophila* Smoothened was used as an outgroup, according to an analysis that had previously defined 3 major Frizzled subfamilies (2). Bayesian analysis was performed by using the MrBayes 3.1.2 program (3). All fixed-rate amino acid models were explored in the MCMC model estimation mode. The fixed-rate amino acid models converged to the WAG model (4) after 1,100 generations; 4 chains were run for 1,000,000 generations; after a burn-in of 250,000 generation every 100th tree was sampled for a 50% majority consensus. Hs, *Homo sapiens*; Nv, *Nematostella vectensis*; Ag, *Anopheles gambiae*; Pv, *Patella vulgata*; Hv, *H. vulgaris*; Hm, *H. magnipapillata*; Che, *Clytia hemisphaerica*; He, *Hydractinia echinata*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*.

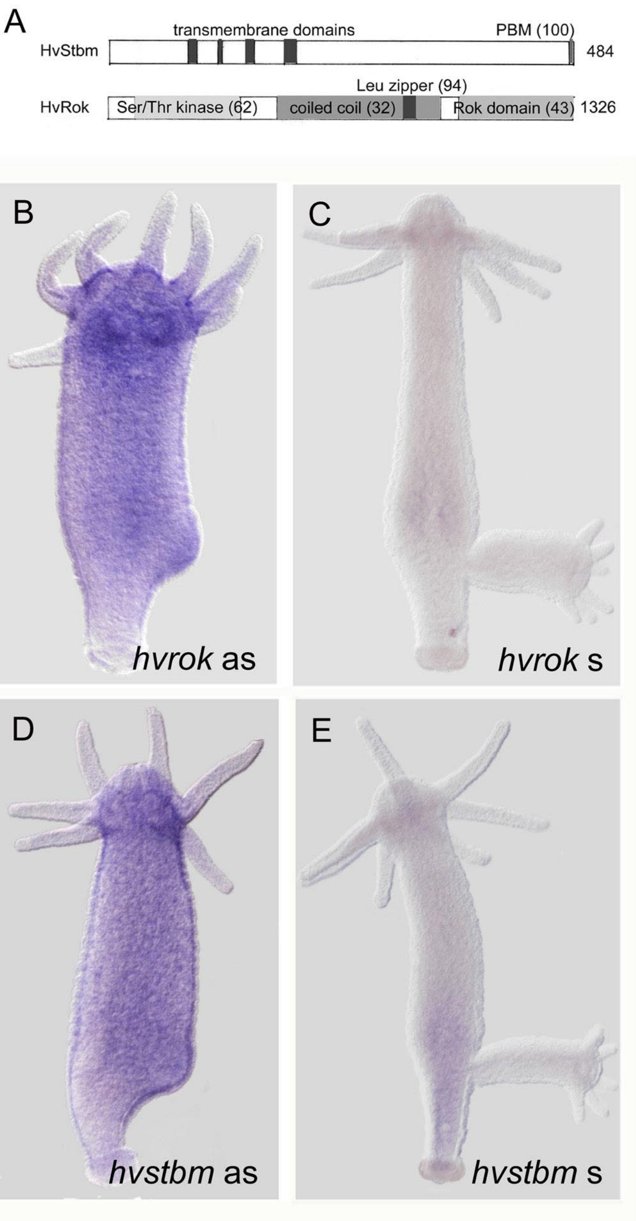

Fig. S3. Characterization of *Hydra Rho-kinase* and *strabismus* orthologs. (*a*) Domain structure of the predicted HvRok and HvStbm proteins. Numbers at the ends of the bars represent the total number of amino acids; numbers in parenthesis show amino acid identity between the *Hydra* and human orthologues in protein–protein interaction domains; (*b–e*) *hvrok* and *hvstbm* genes show rather uniform in situ expression patterns along the body column of intact *Hydra* and during bud formation; as, antisense probes; s, sense control probes.

A

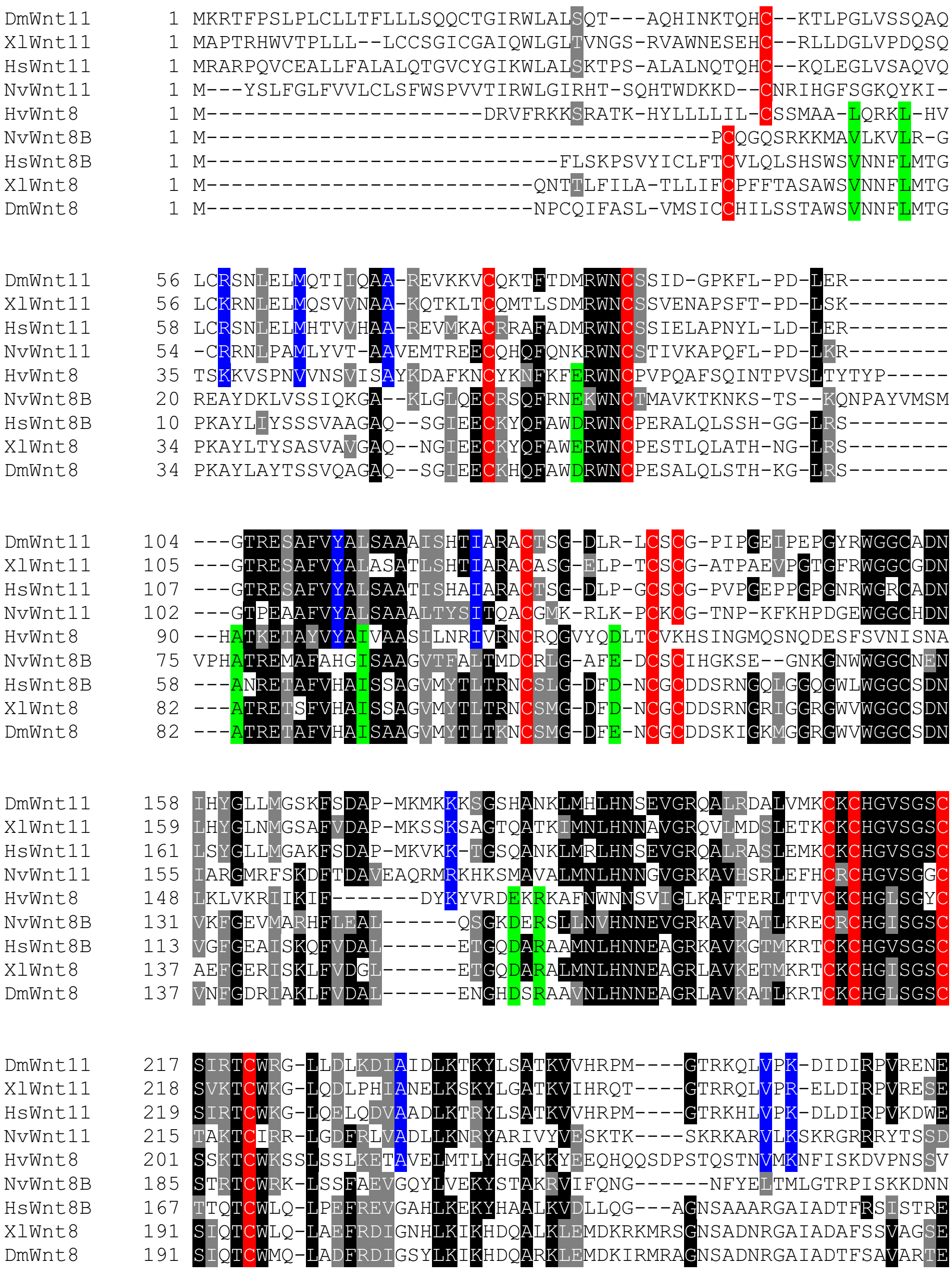

Fig. S4. Amino acid alignments of noncanonical Wnt pathway members. (*A–E*) Amino acid alignments were done using ClustalW (www.ebi.ac.uk/clustalw/) and visualized using the GeneDoc software (www.psc.edu/biomed/genedoc/). Conserved residues are shown with black background; semiconservative substitutions are shown in grey. (*A*) The predicted amino acid sequence of HvWnt8 shows similarity to members of the Wnt11 subfamily. In direct comparison, amino acid residues show identity to or similarity with members of either the Wnt8 (green color) or Wnt11 (blue color) subfamilies. Positions of the conserved cysteine residues are indicated in red. (*C*) Positions of 10 highly conserved cysteine residues specific for the extracellular Frizzled domain are indicated in red. Accession numbers: HvWnt8, AM279158; NvWnt11, AY687349; NvWnt8, AY792510; DmWnt11, NP_571151; DmWnt8, AAC59697; XlWnt11, AAH84745; XlWnt8, CAA40510; HsWnt11, CAA74159; HsWnt8B, CAA71994; HvWnt5, AM263447; NvWnt5, AX725202; HsWnt5A, NM_003392; HsWnt5B, NM_030775; XlWnt5A, P31286; BfWnt5, AF361014; HmFz2, EU442372; NvFz2, XM_001634945; MmFz5, NM_022721; MmFz8, NM_008058; DmFz2, NM_079431; HvRok, AM263448; HsRok1, BAA75636; HsRok2, O75116; XlRok, AAC06351; DmRok, AAF03776; HvStbm, AM263457, HsStbm1, Q9ULK5; HsStbm2, Q8TAA9, XlStbm, AAK70879; DmStbm, AAC02533; PdStbm, CAJ26300. Species abbreviations: Hv, *Hydra vulgaris;* Hm, *Hydra magnipapillata;* Nv, *Nematostella vectensis;* Pd, *Platynereis dumerilii;* Dm, *Drosophila melanogaster;* Bf, *Branchiostoma floridae;* Dr, *Danio rerio;* Xl, *Xenopus laevis;* Mm, *Mus musculus;* Hs, *Homo sapiens.*

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DmWnt11    271 LVYLQSSPDYCMKNDKLGSFGTQDRQCNKTSS-----GSDSCDLMC--CGRGYNPYTERV
XlWnt11    272 LVYLVSSPDYCTKNPKLGSYGTQDRLCNKTSV-----GSDSCNLMC--CGRGYNAYTETI
HsWnt11    273 LVYLQSSPDFCMKNEKVGSHGTQDRQCNKTSN-----GSDSCDLMC--CGRGYNPYTDRV
NvWnt11    270 LVALQGSPNYCHKNRKRGTAGTHGRLCDPTKRR----GEGSCAYLC--CGRGHRTEEVVH
HvWnt8     261 LVYLTESPDYCKKNSSIEVQGTLNRECNHHLD-------DSCKKLCSSCGYRKHSFVKTI
NvWnt8B    238 FIYSESSPDYCQRNMTVGSAGVLGRECEGSKD-----ELVRCRQLCDSCRFDTQEFTEIK
HsWnt8B    223 LVHLEDSPDYCLENKSLGLLGTEGRECLRRGRALGRWELRSCRRLCGDCGLAVEERRAET
XlWnt8     250 LIFLEDSPDYCLKNISLGLQGTEGRECLQSGKNLSQWERRSCKRLCTDCGLRVEEKKTEI
DmWnt8     250 LIFMEDSPDYCVKNLSMGLHGTEGRECLQSGKNLSQWERRSCRRLCHECGLKVEERRIET

DmWnt11    324 VER-CHCKYHWCCYVTCKKCDKTVEKYVCK---------------------
XlWnt11    325 VER-CQCKYHWCCYVMCKKCERTVERYVCK---------------------
HsWnt11    326 VER-CHCKYHWCCYVTCRRCERTVERYVCK---------------------
NvWnt11    324 EER-CECKYIWCCYVKCQTCRKRVRESRCL---------------------
HvWnt8     314 ENMQCNCKFRWCCTVVCEKCVSRQISSRCSLTLR-----------------
NvWnt8B    293 NTF-CNCKFHWCCKVKCMTCKETTRKTRCVARQQAL---------------
HsWnt8B    283 VSS-CNCKFHWCCAVRCEQCRRRVTKYFCSRAERPRGGAAHKPGRKP----
XlWnt8     310 ISS-CNCKFHWCCTVKCEQCKQVVIKHFCARRERDSNMLNTKRKNRGHRR-
DmWnt8     310 VSS-CNCKFHWCCTVKCETCTQTVTRYFCAKRHRNRRPHNHSRKRQHTRRG
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Fig. S4 continued.

B

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HvWnt5     1 M-----------------------------------LTNFISGS----------------
NvWnt5     1 M----------------------------------VLTSFFHAGHSVWSVNIP-------
HsWnt5A    1 M-KKSIGILSPGVALGMAGSAMSSKFFLVALAIFFSFAQVVIEANSWWSLGMNN---PVQ
HsWnt5B    1 M---------------------PSLLLLFTAALLSSWAQLLTDANSWWSLALN----PVQ
XlWNt5A    1 MRKNLWTFQFGGEASGLVGSAMVSQHFVVLLMSLYCLTQSVVESSSWWSLGMN----PVQ
BfWnt5     1 M-----------------AVQVSLRVLRVLVTLLSCYTHLGRVRATWWQMAVDSRLYSLS

HvWnt5    10 ------VFPDIKICDKRRSLTEKQIKLCNTNSDHMAYIIEGRRIAIEECMKQFSDRGWNC
NvWnt5    20 -HQAYIISVQPGLCMNIGGLTREQIDLCQKNIDHMASVGLGAKMAIQECQFQYQYEKWNC
HsWnt5A   57 MSEVYIIGAQP-LCSQLAGLSQGQKKLCHLYQDHMQYIGEGAKTGIKECQYQFRHRRWNC
HsWnt5B   36 RPEMFIIGAQP-VCSQLPGLSPGQRKLCQLYQEHMAYIGEGAKTGIKECQHQFRQRRWNC
XlWNt5A   57 MPEVYIIGAQP-LCSQLSGLSQGQKKLCQLYQDHMQFIGDGAKTGIKECQYQFRHRRWNC
BfWnt5    44 RAELYIIGAQP-LCTTLAGLSSGQRKLCNLYQDHMSSVGIGARQGIEECQHQFRDRRWNC

HvWnt5    64 TFPEPNIIP--FLHPYMPLGTRETAFVHSIIAAATMHSISRACMENKLSSHCSCSQEKKP
NvWnt5    79 SIPDAEKSSLFERITSKDVATREAALTYAISSAGVVWALARACTEGNLST-CSCSRERRP
HsWnt5A  116 STVDNTS----VFGRVMQIGSRETAFTYAVSAAGVVNAMSRACREGELST-CGCSRAARP
HsWnt5B   95 STADNAS----VFGRVMQIGSRETAFTHAVSAAGVVNAISRACREGELST-CGCSRTARP
XlWNt5A  116 STVDNTS----VFGRVMQIGSRETAFTYAISAAGVVNAVSRACREGELST-CGCSRAARP
BfWnt5   103 TTSDEDS----VFGRIVNIGSREASFTYAIAAAGVVNAVSRACREGELTT-CGCSRAKRP

HvWnt5   122 ENLPKTDMWNGCGDNLPYGYQFSKEFVDSKE--TILKDSAFNFGRVLMNLHNNEAGRWAV
NvWnt5   138 LDLNKEYQWGGCGDNIEYAVKFGREFMEAGEDHRPTEEDRKKYARTLMNLHNNNLGRRVV
HsWnt5A  171 KDLPRDWLWGGCGDNIDYGYRFAKEFVDARERERIHAKGSYESARILMNLHNNEAGRRTV
HsWnt5B  150 KDLPRDWLWGGCGDNVEYGYRFAKEFVDAREREKNFAKGSEEQGRVLMNLQNNEAGRRAV
XlWNt5A  171 KDLPRDWLWGGCGDNLDYGYRFAKEFVDAREREKIHQKGSYESSRIMMNLHNNEAGRRAV
BfWnt5   158 KDLNRDWLWGGCGDDVEYGYYFAREFVDAQEKQIIPTPGSQAHARQLMNMHNNEAGRKLT

HvWnt5   180 FEKSKIQCRCHGVSKNCATKTCYRQLSEFKDVGYQLEQLHQSAIHVQLSQSGQKN---DT
NvWnt5   198 KDISVVECKCHGVCGSCNLKTCWRQLVEFREIGNALHDKYDAAVQVALKRKEGRSLLLPM
HsWnt5A  231 YNLADVACKCHGVSGSCSLKTCWLQLADFRKVGDALKEKYDSAAAMRLNSRGK-------
HsWnt5B  210 YKMADVACKCHGVSGSCSLKTCWLQLAEFRKVGDRLKEKYDSAAAMRVTRKGR-------
XlWNt5A  231 STLADVACKCHGVSGSCSLKTCWLQLADFRKVGDHLKEKYDSAGAMKLNTRGK-------
BfWnt5   218 FSNARVACKCHGVSGSCSLKTCWQQLADFRTVGNLLKDKYDGANEVKLIKRGKR------

HvWnt5   237 EITKLVQFNSNIDIYTSKDMIYLDDSPSYCNQNLSIGSFGTEGRECIKDDNSSKECGRLC
NvWnt5   258 RSRHYSQRKAKSAQETRDELVYIDKSPDFCSKNAAHGAQGTRGRKCIKESLGKDGCNLLC
HsWnt5A  284 ----LVQVNSRFNSPTTQDLVYIDPSPDYCVRNESTGSLGTQGRLCNKTSEGMDGCELMC
HsWnt5B  263 ----LELVNSRFTQPTPEDLVYVDPSPDYCLRNESTGSLGTQGRLCNKTSEGMDGCELMC
XlWNt5A  284 ----LVQVNNKFNSPTMNDLVYIDPSPDYCVHNESTGSLGTQGRLCNKTSEGMDGCELMC
BfWnt5   272 --YRLDRRNPRFNVFTDEDLVYLNKSPDYCNADPTIGSLGTHGRECNKTGLGTDGCNLMC

HvWnt5   297 CEKGHYTKKVFTTENCACKFIWCCEVKCQSCKKQQNKHYCK
NvWnt5   318 CSRGYKMKKEVQATRCRCKFHWCCKVKCKTCIKNVTTHICN
HsWnt5A  340 CGRGYDQFKTVQTERCHCKFHWCCYVKCKKCTEIVDQFVCK
HsWnt5B  319 CGRGYNQFKSVQVERCHCKFHWCCFVRCKKCTEIVDQYICK
XlWNt5A  340 CGRGYDQFKTVQTERCHCKFHWCCYVKCKKCTEVVDQFACK
BfWnt5   330 CGRGYNTFKREKVERCNCKFHWCCYVKCKRCRSIKNVYVCK
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Fig. S4 continued.

C

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HmFz2     1 MRRNLYLHILKSVIIHLLCLNLIFCEPR---------------------------GIRERY
NvFz2     1 MAT-RRLDRVFVAVAFISCLN--------------------------------YVLTK-
MmFz5     1 MAR-PDPSAPPSLL--LLLLAQLVG------------------------------RAAAAS
MmFz8     1 MEW-GYLLEVTSLLAALAVLQRSSG------------------------------AAAASA
DmFz2     1 MRH-NRLKVLILGLVLLLTSCRADGPLHSADHGMGGMGMGGHGLDASPAPGYGVPVIPKD

HmFz2    44 ETRKCEKLSITMCEKVGYNLTFMPNKHGHVNQIDAGQLIRQFAPLVAINCSPLLAHFVCS
NvFz2    79 -EMKCEEITIPMCRGIGYNLTYMPNMFNHDTQAEAALEVHQFWPLVEIQCSPDLRFFLCS
MmFz5    48 KAPVCQEITVPMCRGIGYNLTHMPNQFNHDTQDEAGLEVHQFWPLVEIHCSPDLRFFLCS
MmFz8    50 KELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCS
DmFz2    54 PNLRCEEITIPMCRGIGYNMTSFPNEMNHETQDEAGLEVHQFWPLVEIKCSPDLKFFLCS

HmFz2    95 LYAPMCDPVYNKDIVPCQSLCENIRNSCEKFMKQNGYTWPDNLQCSQFPTRE--KDPICM
NvFz2    85 MYAPLCEKHYDKDLPPCRSVCERARTGCAPLMRKYGFSWPERMKCENFPELG-DENTLCM
MmFz5    89 MYTPICLPDYHKPLPPCRSVCERAKAGCSPLMRQYGFAWPERMSCDRLPVLGGDAEVLCM
MmFz8    91 MYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQG-NPDTLCM
DmFz2   120 MYTPICLEDYHKPLPVCRSVCERARSGCAPIMQQYSFEWPERMACEHLPLHG-DPDNLCM

HmFz2   153 KPDFDFPEPSIP-----------------------------------------THLNLP
NvFz2   144 GR--------------------------------------------------NSSGESTPK
MmFz5   149 DYNRSEATTASPKSFPAKPTLPGPPG-------------------------------A
MmFz8   150 DYNRTDLTTAAP-SPPRRLPPPPPPGEQPPSGSGHSRPPGARPPHRGGSSRGSGDAAAAP
DmFz2   179 EQPSYTEAGSGGSSGGSGGSGSGSGSGGKRKQGGSG--------SGGSGAGGSSGSTSTK

HmFz2   171 DENKKTSLIQTKDVFKIRDFSGCGCLCKYPFVSVNSS-----------------------
NvFz2   155 PSQSPQP----APSPDTAAISANCCSCRPPFVSIAGKNIKPTR-----------------
MmFz5   176 PSSGGEC------PSGGPS----VCTCREPFVPILKESHPLYN-----------------
MmFz8   209 PSRGGKAR----PPGGGAAPCEPGCQCRAPMVSVSSERHPLYN-----------------
DmFz2   231 PCRGRNSKNCQNPQGEKASGKECSCSCRSPLIFLGKEQLLQQQSQMPMMHHPHHWYMNLT

HmFz2   208 -SNTDLFPPCVLPCGQYFFSNTQNIFMKFWLSLWSILSLITTAVTLLTFLIDRTKFKFVE
NvFz2   194 AISVGGVPQCAMACNRTYFTHDQDSFASFWIGLWAILCFISTLVTTLTFLVDMHRFKYPE
MmFz5   209 KVRTGQVPNCAVPCYQPSFSPDERTFATFWIGLWSVLCFISTSTTVATFLIDMERFRYPE
MmFz8   248 RVKTGQIANCALPCHNPFFSQDERAFTVFWIGLWSVLCFVSTFATVSTFLIDMERFKYPE
DmFz2   291 VQRIAGVPNCGIPCKGPFFSNDEKDFAGLWIALWSGLCFCSTLMTLTTFIIDTERFKYPE

HmFz2   267 QPIILISFCYFIVSFGYIIRLIYGFKAIACNDHT--------------------------
NvFz2   254 RPIIFLSGCYLMVSVGYIIRLIAGHKQIACDSN---------------------------
MmFz5   269 RPIIFLSACYLCVSLGFLVRLVVGHASVACS------------------------REHS-
MmFz8   308 RPIIFLSACYLFVSVGYLVRLVAGHEKVACSGGAPGAGGRGGAGGAAAAGAGAAGRGASS
DmFz2   351 RPIVFLSACYFMVAVGYLSRNFLQNEEIACDG----------------------------

HmFz2   301 --------------GFLHYASTGPASCTAVFILTYFFTNVSWIWWVVLSINWFLSSGLKW
NvFz2   287 --------------GLMRYDTSGPASCTIVFLLVYFFGMASSVWWVILAFTWFLSAGMKW
MmFz5   304 ---------------HIHYETTGPALCTVVFLLVYFFGMASSIWWVILSLTWFLAAGMKW
MmFz8   368 PGARGEYEELGAVEQHVRYETTGPALCTVVFLLVYFFGMASSIWWVILSLTWFLAAGMKW
DmFz2   383 --------------LLLRESSTGPHSCTLVFLLTYFFGMASSIWWVILSFTWFLAAGLKW

HmFz2   347 TNGTISSYSQYFHFVAWLIPTIQTMAILAMSAIDGDPVSGLCTVGNHDSNTLTIFVIGPL
NvFz2   333 SSEAITNYSQYFHAVAWLVPAIQAIAVLAMSTIDGDPVSGICYVGNHNLQSLVVFVIVPL
MmFz5   349 GNEAIAGYAQYFHLAAWLIPSVKSITALALSSVDGDPVAGICYVGNQNLNSLRGFVLGPL
MmFz8   428 GNEAIAGYSQYFHLAAWLVPSVKSIAVLALSSVDGDPVAGICYVGNQSLDNLRGFVLAPL
DmFz2   429 GNEAITKHSQYFHLAAWLIPTVQSVAVLLLSAVDGDPILGICYVGNLNPDHLKTFVLAPL
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Fig. S4 continued.

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HmFz2    407 LIYTMLSLSFFIAGVVAKLRIEQTIRNEA-----KNNLKSGRFLSRVGMFTLILFVPAVS
NvFz2    393 VVYLVFGTSFLMAGFYSLVRIRKLLRQHG----STKTDKLEKLMIRIGVFSVLYTVPATI
MmFz5    409 VLYLLVGTLFLLAGFVSLFRIRSVIKQGG-----TKTDKLEKLMIRIGIFTLLYTVPASI
MmFz8    488 VIYLFIGTMFLLAGFVSLFRIRSVIKQQGG---PTKTHKLEKLMIRLGLFTVLYTVPAAV
DmFz2    489 FVYLVIGTTFLMAGFVSLFRIRSVIKQQGGVGAGVKADKLEKLMIRIGIFSVLYTVPATI

HmFz2    462 LLGCYFYEHSYKEIWEKSTNCDCMP-----IKKQPIFYIFLFKYLMSLVIGIAIGLGTLN
NvFz2    449 VVACYYYELVNRETWERTINCSGCG--VTR--VKPDHSVFIIKYFMALVVGITSGFWIWS
MmFz5    464 VVACYLYEQHYRESWEAALTCACPGPDAGQPRAKPEYWVLMLKYFMCLVVGITSGVWIWS
MmFz8    545 VVACLFYEQHNRPRWEATHNCPCLRDLQPDQARRPDYAVFMLKYFMCLVVGITSGVWVWS
DmFz2    549 VIGCYLYEAAYFEDWIKALACPCAQ--VKGPGKKPLYSVLMLKYFMALAVGITSGVWIWS

HmFz2    517 SDALGAWRRFFKRHCKITK-------------------------------------SLAQ
NvFz2    505 GKTIESWRNFCARLSGTQN---------------------------------TRRAIPPK
MmFz5    524 GKTLESWRRFTSRCCCSSR---RGHKSGGAMA------AGD-----------------YA
MmFz8    605 GKTLESWRALCTRCCWASKGAAVGAGAGGSGP------GGSGPGPGGGGGHGGGGGSLYS
DmFz2    607 GKTLESWRRFWRRLLGAPDRTGANQALIKQRPPIPHPYAGSGMGMPVGSAAGSLLATPYT

HmFz2    540 EKNGQLIPNNSKKGVAI-----------
NvFz2    532 KANTATV---------------------
MmFz5    558 EASAALTGRTGPPGPTAAYHKQVSLSHV
MmFz8    659 DVSTGLTWRSG-TASSVSYPKQMPLSQV
DmFz2    667 QAGGASVASTSHHHLHHHVLKQPAASHV
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Fig. S4 continued.

D

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HvRok      1 M-------------------TEVEKRYQSLAEKLLKPTNELYVEGLLDTVTSLYYECTSS
HsRok1     1 MSRPPPTGKMPGAPETAPGDGAGASRQRKLEALIRDPRSPINVESLLDGLNSLVLDLDFP
HsRok2     1 MSRPPPTGKMPGAPETAPGDGAGASRQRKLEALIRDPRSPINVESLLDGLNSLVLDLDFP
XlRok      1 M--------------SPRQDEYMGTRWQTLEAILRDPRSPINVEGLLDGLNSIVLDLDFP
DmRok      1 M-----PAGRETVTKQRSMDVERRRRANTLEREMRDPTSICNVDCLLDTVSALVSDCDHE

HvRok     42 HIRNEKNFENFMKRYAAAAEEITKSRINIKDFNEIKVLGQGAFGEVKLMRHKDTKQLYAM
HsRok1    61 ALRKNKNIDNFLNRYEKIVKKIKGLQMKAEDYDVVKVIGRGAFGEVQLVRHKASQKVYAM
HsRok2    61 ALRKNKNIDNFLNRYEKIVKKIRGLQMKAEDYDVVKVIGRGAFGEVQLVRHKASQKVYAM
XlRok     47 ALRKNKNIDNFLNRYEKIVREVRKLQMKAEDYDVVKVIGRGAFGEVQLVRHKSSQKVYAM
DmRok     56 SLRRLKNIEQYAAKYKPLAMQINQLRMNVEDFHFIKLIGAGAFGEVQLVRHKSSSQVYAM

HvRok    102 KLLSKFEMLKKSEVAFFWEERDIMAHANSEWIMAIHYAFQDDKYLYMAMDYMPGGDFVSL
HsRok1   121 KLLSKFEMIKRSDSAFFWEERDIMAFANSPWVVQLFYAFQDDRYLYMVMEYMPGGDLVNL
HsRok2   121 KLLSKFEMIKRSDSAFFWEERDIMAFANSPWVVQLFYAFQDDRYLYMVMEYMPGGDLVNL
XlRok    107 KLLSKFEMIKRSDSAFFWEERDIMAFANSPWVVQLFCAFQDEKHLYMVMEYMPGGDLVNL
DmRok    116 KRLSKFEMMKRPDSAFFWEERHIMAHANSEWIVQLHFAFQDAKYLYMVMDFMPGGDIVSL

HvRok    162 LSNYDIPEDWAAFYIAELVLAIDALHKYGYVHRDIKPDNMLLDKNGHLKLADFGTCIRMD
HsRok1   181 MSNYDVPEKWAKFYTAEVVLALDAIHSMGLIHRDVKPDNMLLDKHGHLKLADFGTCMKMD
HsRok2   181 MSNYDVPEKWAKFYTAEVVLALDAIHSMGLIHRDVKPDNMLLDKHGHLKLADFGTCMKMD
XlRok    167 MSNYDVPEKWAKFYTAEVVLALNAIHSMGLIHRDVKPDNMLLDKYGHLKLADFGTCMKMD
DmRok    176 MGDYDIPEKWAIFYTMEVVLALDTIHNMGFVHRDVKPDNMLLDSYGHLKLADFGTCMRMG

HvRok    222 KDGLVHSDTAVGTTDYLSPEVLLTERHGHGVYGRECDYWAVGVVLYELLVGDPPFLDQSY
HsRok1   241 ETGMVHCDTAVGTPDYISPEVLKSQ-GGDGFYGRECDWWSVGVFLYEMLVGDTPFYADSL
HsRok2   241 ETGMVHCDTAVGTPDYISPEVLKSQ-GGDGFYGRECDWWSVGVFLYEMLVGDTPFYADSL
XlRok    227 QTGMVRCDTAVGTPDYISPEVLKSQ-GGDGYYGRECDWWSVGVFLFEMLVGDTPFYADSL
DmRok    236 ANGQVVSSNAVGTPDYISPEVLQSQ-GVDNEYGRECDWWSVGIFLYEMLFGETPFYADSL

HvRok    282 SGTYEKILNHKNSLQFPTDIEIKSDCKKVICGFLTTREHRLGRNGIDEIKSYKFFQREDW
HsRok1   300 VGTYSKIMDHKNSLCFPEDAEISKHAKNLICAFLTDREVRLGRNGVEEIRQHPFFKNDQW
HsRok2   300 VGTYSKIMDHKNSLCFPEDAEISKHAKNLICAFLTDREVRLGRNGVEEIRQHPFFKNDQW
XlRok    286 VGTYSKIMDHKNSLNFPEDVEISAHAKNLICAFLTDREVRLGRNGIEDIKQHPFFKNDQW
DmRok    295 VGTYGKIMDHKNSLSFPPEVEISEQAKALIRAFLTDRTQRLGRYGIEDIKAHPFFRNDTW

HvRok    342 NWDNIRSNVAKFTPDLDSDIDTRNFDDFSDLEKKNTDTFELSKVFTGNHLPFIGYTYSKH
HsRok1   360 HWDNIRETAAPVVPELSSDIDSSNFDDIEDD-KGDVETFPIPKAFVGNQLPFIGFTYYRE
HsRok2   360 HWDNIRETAAPVVPELSSDIDSSNFDDIEDD-KGDVETFPIPKAFVGNQLPFIGFTYYRE
XlRok    346 NWDNIRETVAPVVPELASDIDTSNFDDIEDD-KGDAETFQIPKAFAGNQLPFVGFTYYRE
DmRok    355 SFDNIRESVPPVVPELSSDDDTRNFEDIERD-EKPEEVFPVPKGFDGNHLPFIGFTYTGD

HvRok    402 NRLIGGNQGTSEENNSK--------VQSLLTKIREVENQLKNEKNAKDESEKVLKNLKNK
HsRok1   419 NLLLSDSPSCRENDSIQSRKNE--ESQEIQKKLYTLEEHLSNEMQAKEELEQKCKSVNTR
HsRok2   419 NLLLSDSPSCRENDSIQSRKNE--ESQEIQKKLYTLEEHLSNEMQAKEELEQKCKSVNTR
XlRok    405 NLLLSESSQNCKEKKILCPTNERAVSTSCKKSINKLEEQLHNEMQTKDELEQKFRAVNLR
DmRok    414 YQLLSSDTVDAESKEANVANSGAASNNHGHGHNHRHRPSNSNELKRLEALLERERGRSEA
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Fig. S4 continued.

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HvRok    454 TQKLTSDWEAEVELRKNGEIKIR----DLERAAALYKHDIKEIQRKLDVETDTKKKFEAK
HsRok1   477 LEKTAKELEEEITLRKSVESALR----QLEREKALLQHKNAEYQRKADHEADKKRNLEND
HsRok2   477 LEKTAKELEEEITLRKSVESALR----QLEREKALLQHKNAEYQRKADHEADKKRNLEND
XlRok    465 LEKIVKELDEEASSRKNIESTTR----QLEREKALLQHKNTEYQRKAENDADKKRSLENE
DmRok    474 LEQQDAGLRQQIELITKREAELQRIASEYEKDLALRQHNYKVAMQKVEQEIELRKKTEAL

HvRok    510 FQELQAKLDSESSTKEDSNKLQKKLIIAERENNDLKEKLRLETEGNIKWKKIENDYRKAQ
HsRok1   533 VNSLKDQLEDLKKRNQNSQISTEKVNQLQRQLDETNALLRTESDTAARLRKTQAESSKQI
HsRok2   533 VNSLKDQLEDLKKRNQNSQISTEKVNQLQRQLDETNALLRTESDTAARLRKTQAESSKQI
XlRok    521 VNSLKDQLEDMKRRNQNSQISNEKMNQLQRQLDEANAQLRTESDAAARLRKTQTEMSKQI
DmRok    534 LVETQRNLENEQKTRARDLNINDKVVSLEKQLLEMEQSYKTETENTQKLKKHNAELDFTV

HvRok    570 AVADHAFKELFEKNKQLGIAKTDIEKELMKVQAALQAETTALKQANDQCRDIEKQNALLK
HsRok1   593 QQLESNNRDLQDKNCLLETAKLKLEKEFINLQSALESERRDRTHGSEIINDLQGRICGLE
HsRok2   593 QQLESNNRDLQDKNCLLETAKLKLEKEFINLQSALESERRDRTHGSEIINDLQGRICGLE
XlRok    581 QQLETNNREFQDKTCMLENAKLKLEKDFINLQSALESERRDRTQGSEVISDLQGRISVLE
DmRok    594 KSQEEKVRDMVDMIDTLQKHKEELGQENAELQALVVQEKNLRSQLKEMHKEAENKMQTLI

HvRok    630 DEVDQLRTKYKSDANAMQKLQDELITVEKSKASILFELKQLKAKYEMEKTNTAKQIRKLS
HsRok1   653 EDLKNGKILLAKVELEKRQLQERFTDLEKEKSNMEIDMTYQLKVIQQSLEQEE------A
HsRok2   653 EDLKNGKILLAKVELEKRQLQERFTDLEKEKSNMEIDMTYQLKVIQQSLEQEE------A
XlRok    641 EDLKKGKELLARADAEKQQLHERLAILEKEKSNMEIDMTYKLKALQQSVEKEE------S
DmRok    654 NDIERTMCREQKAQEDNRALLEKISDLEKAHAGLDFELKAAQGRYQQEVKAHQ------E

HvRok    690 TE---------------KKEKKLSEIQILEKESADIQKEREERIRIESKAANLERLMNDL
HsRok1   707 --EHKATKARLADKNKIYESIEEAKSEAMKEMEKKLLEERTLKQKVENLLLEAEKRCSLL
HsRok2   707 --EHKATKARLADKNKIYESIEEAKSEAMKEMEKKLLEERTLKQKVENLLLEAEKRCSLL
XlRok    695 --EHKATKARLADKNKIYQSIEETKSEAMKDMEKKLQEERVAKQRLENNLLETEKQYSML
DmRok    708 --TEKS----------RLVSREEANLQEVKALQSKLNEEKSARIKADQHSQEKERQLSML

HvRok    735 QLDLKNIKQKNVRLEEEYQASQNKIDSLNSLIQEEIVKRSDIQNELNAVMSDLTTQKTKE
HsRok1   765 DCDLKQSQQKINELLKQKDVLNEDVRNLTLKIEQETQKRCLTQNDLKMQTQQVNTLKMSE
HsRok2   765 DCDLKQSQQKINELLKQKDVLNEDVRNLTLKIEQETQKRCLTQNDLKMQTQQVNTLKMSE
XlRok    753 DCDLKQAKQKINELEALKDKLSEDIKNLTLKAEQETQKRSLSQNDLKMQLQQVNCLKMSE
DmRok    756 SVDYRQIQLRLQKLEGECRQESEKVAALQSQLDQEHSKRNALLSELSLHSSEVAHLRSRE

HvRok    795 QQLKSDCNRILDERKQLQEAYNKLKSASAADDIQMKELQDQLEAEQYFSTLYKTQVRELK
HsRok1   825 KQLKQENNHLMEMKMNLEKQNAELRKERQDADGQMKELQDQLEAEQYFSTLYKTQVRELK
HsRok2   825 KQLKQENNHLMEMKMNLEKQNAELRKERQDADGQMKELQDQLEAEQYFSTLYKTQVRELK
XlRok    813 KQLKQEVNHLTEIKLNLEKQNNELRKERVDADGQMKELQDQLEAEQYFSTLYKTQVRELK
DmRok    816 NQLQKELSTQREAKRRFEEDLTQLKSTHHEALANNRELQAQLEAEQCFSRLYKTQANENR

HvRok    855 EEVDERKKEVQCLQSDIQMVTEERDSLSAQLELALAKAESEELARSIAEEQIYDLEKEKT
HsRok1   885 EECEEKTKLGKELQQKKQELQDERDSLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKI
HsRok2   885 EECEEKTKLGKELQQKKQELQDERDSLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKI
XlRok    873 EECEVKGKMYKEVQQKVQELQDERDSLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKI
DmRok    876 EESAER-------LSKIEDLEEERVSLKHQVQVAVARADSEALARSIAEETVADLEKEKT

HvRok    915 MLELEVKDLLAKNKADSFEKFKKLQEADEKIRVLETELQNEVRKRIESIEQIKTDIVEKK
HsRok1   945 MKELEIKEMMARHKQELTEKDATIASLEETNRTLTSDVANLANEKEELNNKLKDVQEQLS
HsRok2   945 MKELEIKEMMARHKQELTEKDATIASLEETNRTLTSDVANLANEKEELNNKLKDVQEQLS
XlRok    933 MKELEIKEMMARHKQELAEKYATITSLEETNKTLTIDVGNLANEKEDLNNRLKEAHEQIQ
DmRok    929 IKELELKDFVMKHRNEINAKEAALATLKEAENELHKKLGQKAAEYEDLVQQHKKQQEELA
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Fig. S4 continued.

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HvRok    975  --------QNEEIEALKKAVKQEQTLKIQAVNKLAEKMSERKEISSKGKGNKVTSAELKK
HsRok1  1005  RLKDEEISAAAIKAQFEKQLLTERTLKTQAVNKLAEIMNR------KEPVKRGNDTDVRR
HsRok2  1005  RLKDEEISAAAIKAQFEKQLLTERTLKTQAVNKLAEIMNR------KEPVKRGNDTDVRR
XlRok    993  RLKEEENSVVTIKTQFEKQLLTERTLKTQAVNKLAEIMNR------KLPTKRGPDTDVRR
DmRok    989  LMRS---SKDEEITKLLDKCKNEVLLKQVAVNKLAEVMNRRDSDLPKQKNKARSTAELRK

HvRok   1027  KEKENKRLQLLLEQEKNKYQITFTKHQGQNSELNKELEKQKEALTKVMMELESKGMVIEQ
HsRok1  1059  KEKENRKLHMELKSEREKLTQQMIKYQKELNEMQAQIAEESQIRIELQMTLDSKDSDIEQ
HsRok2  1059  KEKENRKLHMELKSEREKLTQQMIKYQKELNEMQAQIAEESQIRIELQMTLDSKDSDIEQ
XlRok   1047  KEKENRKLQLDLKSEREKFTQLVIKYQREMNDMQAQIADENQVRIELQMALDSKDSDIEQ
DmRok   1046  KEKEMRRLQQELSQERDKFNQLLLKHQ----DLQQLCAEEQQLKQKMVMEIDCKATEIEN

HvRok   1087  LQEDNKNLNTEIENLRALVPVGTNSSILHPANMKLEGWLSIP-ERRVKKNMLWKKQYVVV
HsRok1  1119  LRSQLQALHIGLDSSSIGSGPGDAEADDGFPESRLEGWLSLP-VRNNTKKFGWVKKYVIV
HsRok2  1119  LRSQLQALHIGLDSSSIGSGPGDAEADDGFPESRLEGWLSLP-VRNNTKKFGWVKKYVIV
XlRok   1107  LR----SQMLGLDSTSIGSGHGDTDAEDGFPESRLEGWLSLP-LRN-AKKFGWNKKYVVV
DmRok   1102  LQSKLNETASLSSADNDPEDSQHSSLLSLTQDSVFEGWLSVPNKQNRRRGHGWKRQYVIV

HvRok   1146  SQQKIFFFTNEQDKA-PNTPAMILDIGKLFHVRSVTQGDVIRVDVKDIPKIFQILYANEG
HsRok1  1178  SSKKILFYDSEQDKE-QSNPYMVLDIDKLFHVRPVTQTDVYRADAKEIPRIFQILYANEG
HsRok2  1178  SSKKILFYDSEQDKE-QSNPYMVLDIDKLFHVRPVTQTDVYRADAKEIPRIFQILYANEG
XlRok   1161  SSRKILFYDSEQDKE-LSNPSMVLDIDKLFHVRPVTQTDVYRADAKEIPRIFQILYANEG
DmRok   1162  SSRKIIFYNSDIDKHNTTDAVLILDLSKVYHVRSVTQGDVIRADAKEIPRIFQLLYAGEG

HvRok   1205  ESKNPEEKTE-----QDQLEQDKAAVVIPFKDHQFVVMHYHMPNACDMCQRQMWHMIKPP
HsRok1  1237  ESKKEQE--------FPVEPVGEKSNYICHKGHEFIPTLYHFPTNCEACMKPLWHMFKPP
HsRok2  1237  ESKKEQE--------FPVEPVGEKSNYICHKGHEFIPTLYHFPTNCEACMKPLWHMFKPP
XlRok   1220  ESKKEQE--------FQVDPLE-KSNYICHKGHEFIPTLYHFPTSCDACMKPLWHMFKPP
DmRok   1222  ASHRPDEQNQLDVSVLHGNCNEERPGTIVHKGHEFVHITYHMPTACEVCPKPLWHMFKPP

HvRok   1260  LAVECRRCRVKCHKDHVDEEEDVIQPCKVTVDLATAKDLLILANDVDEQKNGYKNYLKKL
HsRok1  1289  PALECRRCHIKCHKDHMDKKEEIIAPCKVYYDISTAKNLLLLANSTEEQQKWVSRLVKKI
HsRok2  1289  PALECRRCHIKCHKDHMDKKEEIIAPCKVYYDISTAKNLLLLANSTEEQQKWVSRLVKKI
XlRok   1271  AALECRRCHIKCHKDHMDKKEEIIAPCKVNYDISTAKNLLLLANSTEEQQKWVSRLVKKI
DmRok   1282  AAYECKRCRNKIHKEHVDK-HDPLAPCKLNHDPRSARDMLLLAATPEDQSLWVARLLKRI

HvRok   1320  FAKVLEP--------------------------------------------
HsRok1  1349  PKKPPAPDPFARSSPRTSMKIQQNQSIRRPSRQLAPNKPS-----------
HsRok2  1349  PKKPPAPDPFARSSPRTSMKIQQNQSIRRPSRQLAPNKPS-----------
XlRok   1331  PKKPPASEHQARSSPRPPAKASLNQSMRRPSRQLPPNKPS-----------
DmRok   1341  QKSGYKAASYNNNS-TDGSKISPSQSTRSSYKPYAVNVQRSATLPANSSLK
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Fig. S4 continued.

E

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HvStbm     1 M--------------------------------ADDLDDGVIEVQVIPQ-----------
HsStbm1    1 MDTESQYSGYSYKSGHSRSSRK--HRDRRDRHRSKSRDGGRGDKSVTIQAPG-EPLLDNE
HsStbm2    1 MDTESTYSGYSYYSSHSKKSHRQGERTRERHKSPRNKDGRGSEKSVTIQPPTGEPLLGND
XlStbm     1 MDNDSQYSGYSYKSGQSRSSRK--HRDRRERHRSKSREGSRGDKSVTIQAPG-EPLLDNE
DmStbm     1 MENESVKSEHSGRSRRSRNHNNNGGGGGGGGGVSGGGGGSVNNGYHRERDRSRHSHRSTH
PdStbm     1 MDTESVRSGRSERSERSQRSNRPYRNKSGRHSSRERSHDRHRERHHGDNHRNGGGHDDTD

HvStbm    18 -----------------------------------------DDNWGETNTITETATSVCT
HsStbm1   58 STRGDER----------------------------------DDNWGETTTVVTG-TSEHS
HsStbm2   61 STRTEEVQ---------------------------------DDNWGETTTAITG-TSEHS
XlStbm    58 STRGEDR----------------------------------DDNWGETTTVVTG-TSEHS
DmStbm    61 SSKSAKGFQRGDMAPYQTSVNMTGDGSHDGQEVIEVQILPQDENWGENTTAVTGNTSEQS
PdStbm    61 RDDRSVTIAPLPRSHHHHSDVTVTQG-RNGEERIEVQIMPQDENWGETTTAITGNTSETG

HvStbm    37 DLEDVDMGLFDDDDVEHRNFSCLNFKQTLSIIFSIGLGIFAILSPIAFLVMPNLISNWK-
HsStbm1   83 ISHDDLT-RIAKDMEDSVPLDCS---RHLGVAAGATLALLSFLTPLAFLLLPPLLWREE-
HsStbm2   87 ISQEDIA-RISKDMEDSVGLDCK---RYLGLTVASFLGLLVFLTPIAFILLPPILWRDE-
XlStbm    83 ISHDDIT-RITKDMEDSAKLDCS---RHLGVVIAGALALLSFLTPIAFMLLPQILWRED-
DmStbm   121 ISMEDINNMWHRESDKGFSFACR---RYVESSFYFLLGCGAFFSPVAMVVMPYVGFFPSA
PdStbm   120 FSMEDMS-RINKEMEEGIGFNCE---RYMGSIVAGMLGVLAFLSPIAMVILPKLGLEEWE

HvStbm    96 -------------TDSCAMTCGGMYISIGVKELVLLVGIWALYFRPTKAFMPRIDVFKVG
HsStbm1  138 -------------LEPCGTACEGLFISVAFKLLILLLGSWALFFRRPKASLPRVFVLRAL
HsStbm2  142 -------------LEPCGTICEGLFISMAFKLLILLIGTWALFFRKRRADMPRVFVFRAL
XlStbm   138 -------------LEQCGTACEGLFISVAFKLLILLLGSWALFFRRPKAFFPRVFVFRAL
DmStbm   178 FDHPEITQTVRTQLLACSEQCKGQLVSLAARLLLLAIGLWAVFMRRTSATMPRIFLYRAL
PdStbm   176 QDP-------------CGPECEGLLISFGSKLFILLIGTWALFFRRPKATMPRIFIFRAV

HvStbm   143 MLSIGYLVVTLFWLFYVIQIFG---------KKSSDLSGIVSFAIYFIDSMLFLHYLALL
HsStbm1  185 LMVLVFLLVVSYWLFYGVRILD---------ARERSYQGVVQFAVSLVDALLFVHYLAVV
HsStbm2  189 LLVLIFLFVVSYWLFYGVRILD---------SRDRNYQGIVQYAVSLVDALLFIHYLAIV
XlStbm   185 LMVLVFLLVVSYWLFYGVRILE---------SRDKNYQGIVQYAVSLVDALLFVHYLAVV
DmStbm   238 VLLLVTICTFAYWLFYIVQVTNGAKIVVETGGDAVDYKSLVGYATNFVDTLLFIHYVAVV
PdStbm   223 ILFLVFIFTFAFWLFYGFRIYK---------AKEKSYHNIVLYAVSLVDTLLFIHYIAII

HvStbm   194 LMWIRHQENIYNVSVIRNVDGSRKHYMIGQCSIQKAAVNVLEKYYIDFNEYNPYLPRPTS
HsStbm1  236 LLELRQLQPQFTLKVVRSTDGASRFYNVGHLSIQRVAVWILEKYYHDFPVYNPALLNLPK
HsStbm2  240 LLELRQLQPMFTLQVVRSTDGESRFYSLGHLSIQRAALVVLENYYKDFTIYNPNLLTASK
XlStbm   236 LLELRQLQPQFTIKVVRSTDGASRFYNIGHLSIQRVAVWILENYYHDFPVYNPALLNLPK
DmStbm   298 LLELRHQQPCYYIKIIRSPDGVSRSYMLGQLSIQRAAVWVLQHYYVDFPIFNPYLERIPI
PdStbm   274 LLEVRQLQPQYAVRITRSPDGESHNYTAGQLSIQRLAVWCLEQYYRDFQVYNPYLEHVSR

HvStbm   254 RSKINKFSNIKFYDLDNKMDMGNGKNFSQQASKAVIAAAALGRRKEGRNDRFYEELEIDR
HsStbm1  296 SVLAKKVSG----FKVYSLGEENSTNNSTGQSRAVIAAAAR-RRDNSHNEYYYEEAEHER
HsStbm2  300 FRAAKHMAG----LKVYNVD--GPSNNATGQSRAMIAAAAR-RRDSSHNELYYEEAEHER
XlStbm   296 SILSKKMSG----FKVYSLGEENNTNNSTGQSRAVIAAAAR-RRDNSHNEYYYEEAEHER
DmStbm   358 SVSKSQRNKISNSFKYYEVDGVSNSQQ-QSQSRAVLAANAR-RRDSSHNERFYEEHEYER
PdStbm   334 RVPKLSG------FKVYDIDGVQGSQTPATRSRAIFAAAAR-RRDQSHNDRFYEEQDYER
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Fig. S4 continued.

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HvStbm    314 RVRKRKARLVAAAEEAFGHIARLNAFDTSKKANGSMNPDEAAQAIFPTLARPLQKYLRTT
HsStbm1   351 RVRKRRARLVVAVEEAFTHIKRLQEEEQ-KNPREVMDPREAAQAIFASMARAMQKYLRTT
HsStbm2   353 RVKKRKARLVVAVEEAFIHIQRLQAEEQQKAPGEVMDPREAAQAIFPSMARALQKYLRIT
XlStbm    351 RVRKRKARLVVAVEEAFTHIKRLQDEDP-KNPREIMDPREAAQAIFASMARAMQKYLRTT
DmStbm    416 RVKKRRARLITAAEEAFTHIKRIHNEPA---PALPLDPQEAASAVFPSMARALQKYLRVT
PdStbm    387 RVRKRKARLFTAAEEAFTHIKRMQEESG---PAIPMDPLEAAQAIFPSMARALQKYLRIT

HvStbm    374 RQQLHYPLESIMKHLAHCIMFDMSARAFLERYTCDQPCVGY-VSSSQSQDWTLISDFAAT
HsStbm1   410 KQQPYHTMESILQHLEFCITHDMTPKAFLERYLAAGPTIQYHKERWLAKQWTLVSEEPVT
HsStbm2   413 RQQNYHSMESILQHLAFCITNGMTPKAFLERYLSAGPTLQYDKDRWLSTQWRLVSDEAVT
XlStbm    410 KQQPYHTMESILQHLEFCITHDMTPKAFLERYLGPGPTIQYHKDRWLAKQWTLVSEEPVT
DmStbm    473 RQQPRHTFESILKHLAHCLKHDLSPRAFLEPYLTESPVMQSEKERRWVQSWSLICDEIVS
PdStbm    444 RQQPRYTMENILQHLATCISCDQTPRAFLERYLTQGPVIWNDKDLRSTQTWVLICDQLLS

HvStbm    433 KQLSDGMIFQLQQNDISLVVVVSRTPIFKISEHAYDIDTNRFILRLSSETSV
HsStbm1   470 NGLKDGIVFLLKRQDFSLVVSTKKVPFFKLSEEFVDPKSHKFVMRLQSETSV
HsStbm2   473 NGLRDGIVFVLKCLDFSLVVNVKKIPFIILSEEFIDPKSHKFVLRLQSETSV
XlStbm    470 NGLKDGVVFELKRQDFSLVVSTKKIPFFKLSEEFVDPKSHKFVMRLQSETSV
DmStbm    533 RPIGNECTFQLIQNDVSLMVTVHKLPHFNLAEEVVDPKSNKFVLKLNSETSV
PdStbm    504 RAVKDGTVFQLRQGDVTLLIAVRHLPHFNVTEEVIHPKNNKFVLRLNSETSV
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Fig. S4 continued.

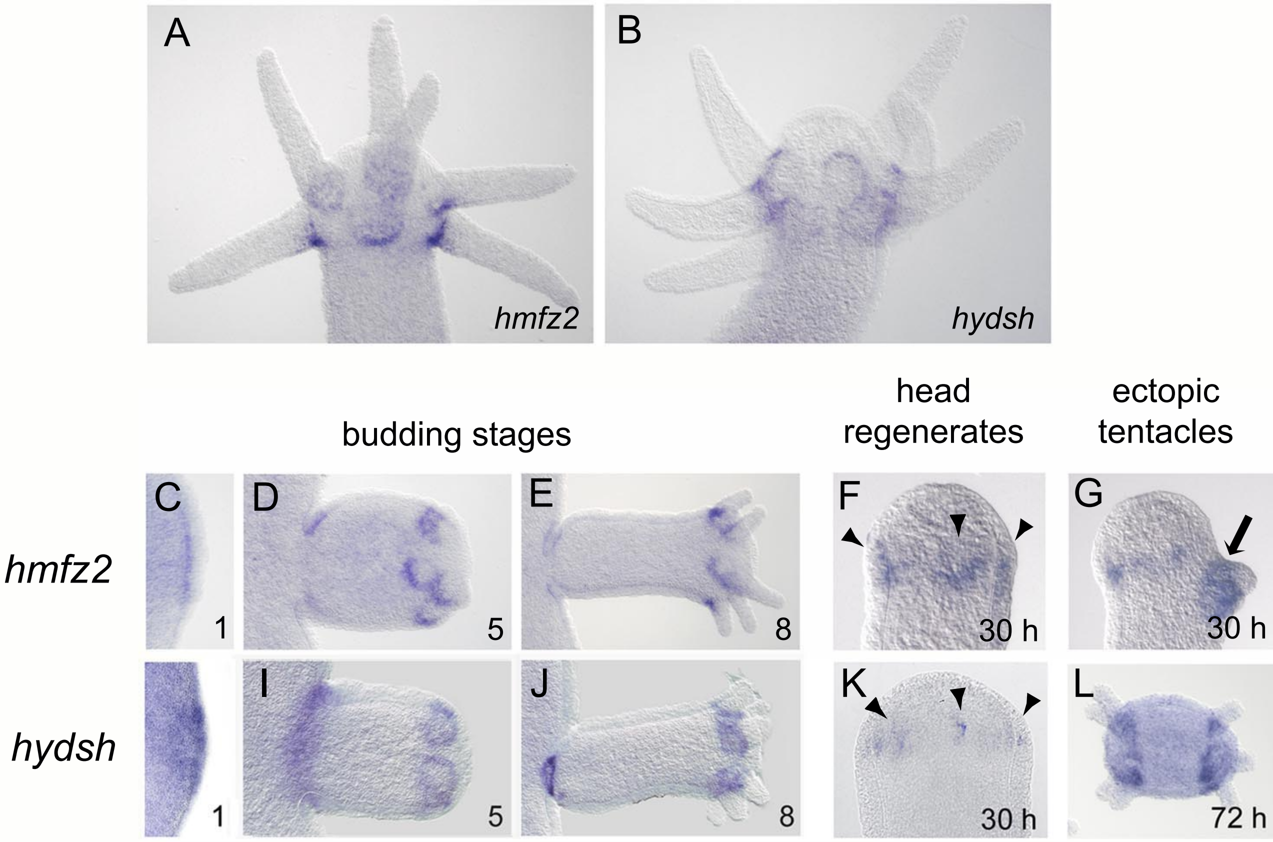


Fig. S5. Gene expression patterns of *hmfz2* and *hydsh* during tentacle and bud evagination. (*a–l*) Spatial and temporal expression patterns of *hmfz2* and *hydsh* are equivalent to the expression pattern of *hvwnt8* during tissue evagination (Fig. 2). (*a* and *b*) Expression of *hmfz2* and *hydsh* at the basis of tentacles in intact *Hydra* heads. (*c–e and h–j*) Expression of *hmfz2* and *hydsh* in the pallisading zone of early bud stages (*c* and *h*) and thereafter in those epithelial cells undergoing shape changes at the basis of evaginating tentacles and buds (*d, e, i,* and *j*). (*f* and *k*) Local up-regulation (arrow heads) of *hmfz2* and *hydsh* during initiation of tentacle regeneration in head regenerates. (*g* and *l*) Up-regulation of *hmfz2* and *hydsh* during the formation of ectopic body tentacles (arrow) in a wild-type head regenerate (*g*), and in an irregularly regenerating tissue piece from a multiheaded mutant (*l*). Numbers represent bud stages or regeneration times.

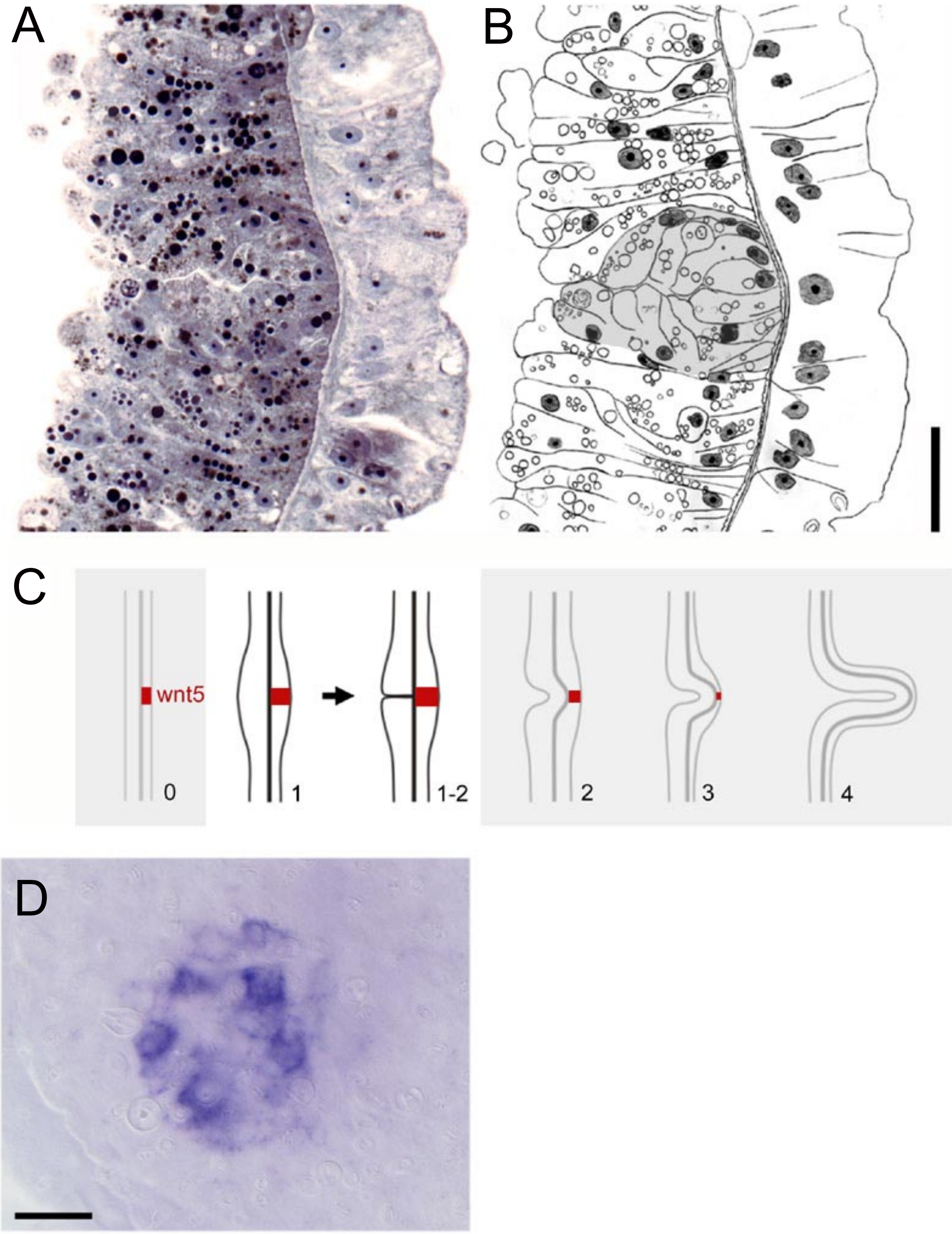


Fig. S6. Symmetry-breaking cell shape changes in the endodermal epithelium during bud initiation. (*a*–*c*) Gelei initially described those cell shape changes representing the first morphological sign of bud evagination (5). In a cluster of endodermal epithelial cells in the centre of the pallisading zone (shaded area in *b*), the cells substantially decrease their distal proximal diameter and increase their basal surface area. This leads to a symmetry-breaking curvature of the muscle layers toward the ectodermal side. (*a*) Histological 3-μm sagittal section from the pallisading area of a stage1–2 bud. (*b*) Schematic representation of this section outlining epithelial cell membranes, cell nuclei, and food vacuoles in the endodermal layer. (*c*) Schematic representation of the position of *hvwnt5*-expressing ectodermal epithelial cells throughout the early budding stages. (*d*) Magnified view of a *hvwnt5*-expressing cell cluster before evagination. In situ hybridization showing a representative *hvwnt5* expressing cluster of roughly 10 ectodermal epithelial cells just before tentacle evagination in a stage 5 bud. Initiation of tentacle and bud evagination occurs in a community, where strongly expressing *hvwnt5* cells are separated by cells exhibiting lower level of *hvwnt5* expression. Such 2D patterns have been theoretically explained by activation-inhibition systems, where several local activation maxima appear close to one other with initially small inhibition originating from each activation peak (6). (Scale bar, 20 μm.)

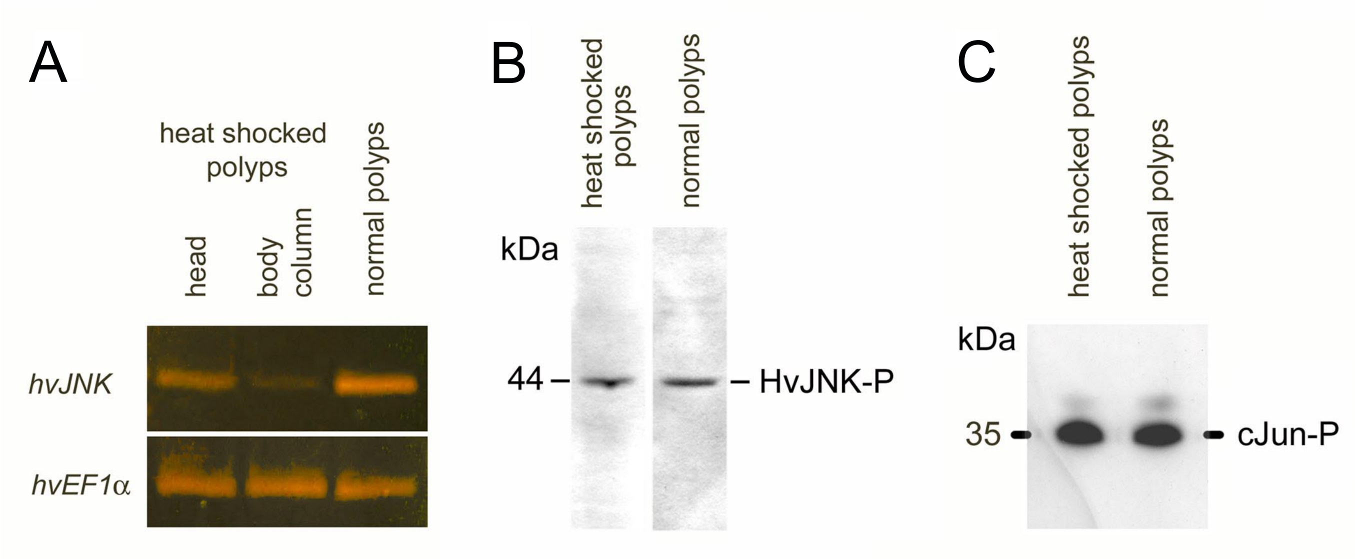
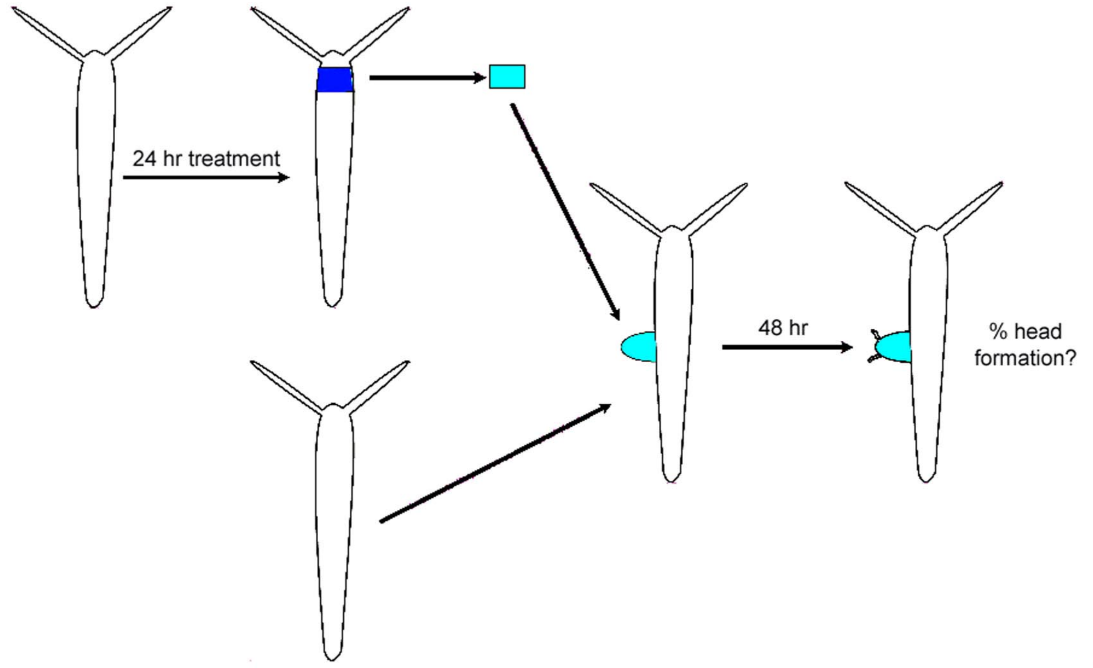


Fig. S7. Detection of JNK activity in the epithelium of *Hydra.* (*a*) Previously, strong *hvJNK* expression had been described in nests of differentiating nematocytes throughout the gastric region of *Hydra* and had not been detected in epithelial cells (7). Here, by using interstitial cell-free *Hydra, hvJNK* mRNA and JNK kinase activity were detected in the epithelial cell lineages. *H. magnipapillata* mutant strains sf-1 and A-10 were used due to their temperature-sensitive interstitial stem cell system. To eliminate all *hvJNK*-expressing nematocyte nests, polyps were incubated at 25 °C for 4 days and then transferred back to 18 °C for three more days under daily feeding. Total loss of interstitial stem cells and *hvJNK*-expressing nematocyte nests was controlled by maceration preparation and in situ hybridization. To detect *hvJNK* mRNA, RT-PCR was done by using RNA from head and gastric tissue of i-cell free *Hydra* and of normal nonshocked controls. Contamination from nuclear DNA is excluded by the presence of large introns in the genomic sequence between the primer pairs. PCR primers: JNK_F-RT2, GGAATGGGATACTCTGAAAACG; JNK_R-RT2, GTCGATTGGCACAGTACATACG; EF1a-Forw, GTATGGTTGCTTCTGACAGC; EF1a-Rev, TGTGAGCAGTGTGACAATCC. PCR conditions: 1 cycle, 95 °C for 3 min; 30 cycles, 95 °C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min; 1 cycle, 72 °C for 2 min. (*b*) Detection of *Hydra* phosphoJNK was done by standard Western blotting using intact polyp tissues lysed in NuPAGE LDS sample buffer (Invitrogen) containing 1:100 phosphatase inhibitor (Sigma-Aldrich) and the phospho-SAPK/JNK (Thr-183/Tyr-185) antibody (9251, Cell Signaling Technology). (*c*) To detect HvJNK kinase activity in cell lysates from i-cell-free *Hydra*, a nonradioactive JNK Assay Kit (9810, Cell Signaling Technology) was used, which visualizes c-Jun phosphorylation in immunoblots. All procedures were carried out according to the kit protocol. It should be noted that the amounts of phospho-c-Jun detected were limited by the detection system and therefore do not represent JNK activity in a quantitative manner.



treatment	no. 2nd axes / no. grafts	2nd axis formation[%]
untreated controls	28/44	64%
25µM SP600125	29/44	66%
25µM ZTM000990	8/44	18%

Fig. S8. Effects of inhibitor treatments on the capacity for head activation. Treatment with the β-Catenin-specific inhibitor ZTM000990 strongly reduced the capacity of tissue pieces to induce a secondary axis, whereas treatment with the JNK-specific inhibitor SP600125 had no effect. Animals were treated with 25 μM of either SP600125 or ZTM000990 for 1 day. Then, tissue pieces of about 1/8 of total body size were excised directly below the heads and transplanted into the middle of the gastric region of untreated hosts according to MacWilliams (8). Two days after successful grafting, transplants were assayed for the induction of secondary axes.

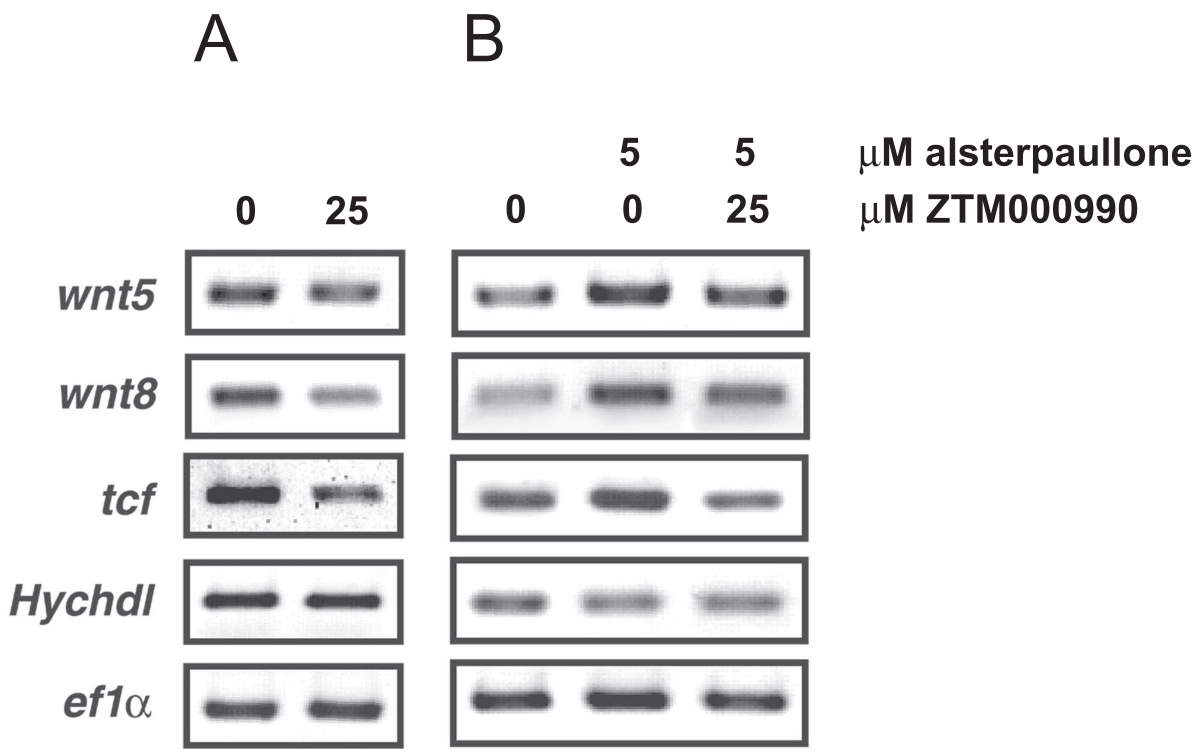


Fig. S9. Detection of the beginning of gene expression responses to alsterpaullone and ZTM000990 during the early phases of treatments. (*a* and *b*) RT-PCR-based detection of early gene expression responses following treatment with the GSK3 inhibitor alsterpaullone and the β-Catenin inhibitor ZTM000990. (*a*) In intact *Hydra* treated with 25 μM ZTM000990, an initial decrease in the expression of *hvwnt5*, *hvwnt8*, and *hytcf* genes was detected after 48 h. Notably, no inhibitory effect on expression of the head organizer-specific chordin-like gene, *Hychdl* (9), was found. (*b*) In intact *Hydra* treated with 5 μM alsterpaullone, *hvwnt5*, *hvwnt8*, and *hytcf* genes started to show up-regulation after 24 h. This stimulation was suppressed, when the polyps were cotreated with 5 μM alsterpaullone and 25 μM ZTM000990. Again, *Hychdl* gene expression levels were unaffected. *Hydra ef1α* gene expression levels were used to evaluate the amounts of target cDNA in both datasets.

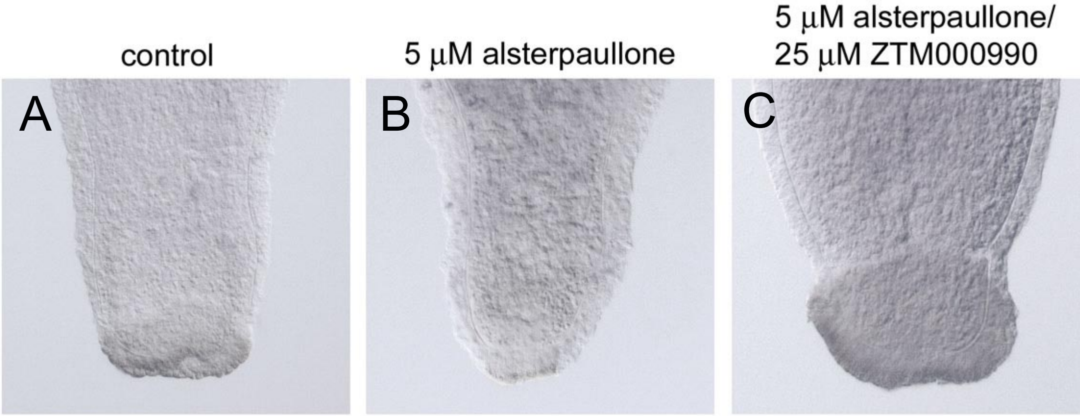


Fig. S10. Treatments with alsterpaullone and ZTM000990 affect foot patterning in *Hydra.* (*a–c*) Differentiation of foot-specific basal disk cells is altered depending on activation or inhibition of Wnt/β-Catenin signaling; β-Catenin stabilization by treatment with 5 μM alsterpaullone results in a reduction of basal dics cells, while cotreatment with 5 μM alsterpaullone and 25 μM of the β-Catenin inhibitor ZTM000990 increases the amount of basal disk cells. Phenotypes were observed 60 h after the onset of treatment. Amino acid alignments were done by using ClustalW (www.ebi.ac.uk/clustalw/) and visualized by using the GeneDoc software (www.psc.edu/biomed/genedoc/). Conserved residues are shown with black background; semiconservative substitutions are shown in gray. (*a*) The predicted amino acid sequence of HvWnt8 shows similarity to members of the Wnt11 subfamily. In direct comparison, amino acid residues show identity to or similarity with members of either the Wnt8 (green color) or Wnt11 (blue color) subfamilies. Positions of the conserved cysteine residues are indicated in red. (*c*) Positions of 10 highly conserved cysteine residues specific for the extracellular Frizzled domain are indicated in red. Accession numbers: HvWnt8, AM279158; NvWnt11, AY687349; NvWnt8, AY792510; DmWnt11, NP_571151; DmWnt8, AAC59697; XlWnt11, AAH84745; XlWnt8, CAA40510; HsWnt11, CAA74159; HsWnt8B, CAA71994; HvWnt5, AM263447; NvWnt5, AX725202; HsWnt5A, NM_003392; HsWnt5B, NM 030775; XlWnt5A, P31286; BfWnt5, AF361014; HmFz2, EU442372; NvFz2, XM_001634945; MmFz5, NM_022721; MmFz8, NM_008058; DmFz2, NM_079431; HvRok, AM263448; HsRok1, BAA75636; HsRok2, O75116; XlRok, AAC06351; DmRok, AAF03776; HvStbm, AM263457, HsStbm1, Q9ULK5; HsStbm2, Q8TAA9, XlStbm, AAK70879; DmStbm, AAC02533; PdStbm, CAJ26300. Hv, *H. vulgaris*; Hm, *H. magnipapillata*; Nv, *N. vectensis*; Pd, *Platynereis dumerilii*; Dm, *D. melanogaster*; Bf, *Branchiostoma floridae*; Dr, *Danio rerio*; Xl, *Xenopus laevis*; Mm, *M. musculus*; Hs, *H. sapiens*.