Gone with the *Wnts*….

The Wnt Signaling Pathway in *Pleurobrachia bachei*

Rebecca Bruders[1,2], Billie J. Swalla[1,2], Leonid Moroz[1,3], and Andrea Kohn[1,3]

Marine Genomics Research Apprenticeship
Spring 2012

[1] University of Washington, Friday Harbor Laboratories, Friday Harbor, WA 98250
[2] Department of Biology, University of Washington, Seattle, WA 98195
[3] Department of Biology, University of Floridam The Whitney Laboratory for Marine Bioscience, St. Augustine, FL 32080

Contact Information:
Rebecca Bruders
Biology Department
University of Washington
bruder2@u.washington.edu
Key Words: *Pleurobrachia bachei*, *Wnt* signaling, development, neural signaling

Abstract:

Wnt signaling is known to be critical for proper embryonic development in most animals studied to date[15]. But key evolutionary questions on the origin and evolution of this pathway in the metazoan common ancestor are still unresolved. Recently, the genome of *Pleurobrachia bachei*, a member of the early branching metazoan linage ctenophora, has been sequenced. Insights into the function of the *Wnt* pathway in *P. bachei* will provide information on early evolution of this key pathway. Three *Wnt* ligand genes were identified in *P. bachei* and cloned for *in situ* hybridization. These genes showed expression in the combs, tentacles, mouth, ciliated grooves and polar fields of the adult *P. bachei*. In a genomic search for other members of the canonical *Wnt* pathway, components of the destruction complex and antagonists were incomplete or missing from the genome. *Wnt* expression in the adult *P. bachei* indicates that *Wnt* could also be playing a role in neurotransmission in the adult.

Introduction:

The phylum Ctenophora, consists of a group of marine dwelling "comb-bearers", the phylum is relatively small containing a estimated 100- 200 different species[1]. Ctenophores are biradially symmetric around the oral-aboral axis. Ctenophores bodies are gelatinous; their most distinct features include the comb rows, which are 8 rows of comb plates, which are ciliated and used for movement. Another notable feature is the apical sensory organ at the aboral end of the ctenophore, which is used in order to detect direction using gravitational force[2]. Many species of adult ctenophores have 2 pairs of long tentacles used to grab pray such as copepods for consumption. A recent debate over the phylogenetic placement of early metazoans has created two major phylogenetic hypotheses. One hypothesis places ctenophores as most basal and one placing sponge most basal as the sister group to all other metazoans [3,4]. Genomes of two well-studied species of ctenophores, *Mnemiopsis leidyi* and *Pleurobrachia bachei* have recently been sequenced. This has given us the opportunity to study and ask complex questions on the early evolution of important signaling pathways. One such pathway is the *Wnt* signaling pathway in *P. bachei*.

The *Wnt* signaling pathway is involved early in development in body patterning, often in gastrulation, germ layer specification and axial polarity and it is also involved in patterning of other body parts such as limbs later in development[5]. The *Wnt* pathway may have been essential in the ancestral axial patterning system [6] that likely evolved shortly prior to the divergence of the metazoans[7]. The *Wnt* signaling pathway (Figure 1A

/B) involves the Wnt ligand interacting with a transmembrane receptor frizzled. Once the Wnt ligand binds to a frizzled receptor protein and its co-receptor Low-density lipoprotein receptor-related protein (LRP), Dishevelled is then activated; Dishevelled inhibits Glycogen Synthase Kinase-3 (GSK-3), an enzyme that prevents ß-catenin from dissociation from the adenomatous polyposis coli (APC) complex. This complex then degrades ß-catenin. If ß-catenin is released from the APC complex it can enter the nucleus where it interacts with LEF or TCF DNA binding proteins and activates specific genes. When Wnt is present, this pathway is active and Wnt responsive genes are turned on and transcription occurs. If Wnt antagonists such as Dickkopf, secreted frizzled, Wnt inhibitory factor, and Cerberus are present, Wnt binding is inhibited and the Wnt responsive genes are not transcribed.

Humans have 19 known *Wnt* genes[8] but in a recent study on the ctenophore *M. leidyi* only 4 *Wnt* genes were found in the genome of this basal metazoan and many other known components of the *Wnt* pathway were missing. Genes missing include antagonists to the Wnt pathway such as *Dikkopf, Wnt Inhibitory Factor and Cerberus*. Also found missing in *M. leidyi* was *Axin* a key member of the destruction complex[9].

Methods:

Sampling and Handling:

All *P. bachei* adults were collected off the dock at Friday Harbor Labs, Friday Harbor Washington between the months of April and May 2012. Collected animals were housed in plastic containers with mesh sides in circulating sea water tables prior to fixation.

Embryos were collected from ctenophores placed in spawning jars overnight and collected the fallowing morning.

RNA Probes:

Sequences were chosen via gene prediction by blasting *Mnemiopsis leidyi* Wnt sequences against the recently sequences *P.Bachei* genome. PCR using adult *P. bachei* cDNA libraries made with Smart PCR cDNA Synthesis Kit by BD Biosciences and superscript III by invitrogen. Products were band isolated using E-gel and cloned using the TOPO kit by Invitrogen. Plasmids were then purified using the miniprep kit by quiagen. Dioxigen- labeled anti-sense RNA probes were synthesized at the Whitney Laboratory for Marine Bioscience, a University of Florida Institute.

PCR:

PCR Primer Sequences:
PbWnt 4-1
5'-ATG TAT CAA CTT TTA TCA GTC TTT G -3'
PbWnt4-2
5'- CCC ATC CTT CTT GGC GAT GA-3'

PbWnt 6-1
5'-ATG CGA CGA TAC CTC AAA TCT ATA C-3'
PbWnt 6-2
5'- CTA CAT ACA GAA TGC TTC TTG GA -3'

PbWnt9-3
5'-CCA ATC GCT CAC TCT GCA GGA A-3'
PbWnt9-4
5'-GGG CTG AGT GGT GTT ACA TCT-3'

In Situ probe sequences

PbWnt6
ATGCGACGATACCTCAAATCTATACATGTNATGGATGTGGGATACCGGAAAT
GGCTCCTCTACGATCAGAATGATTTCCAACCTTCACCATCTGACCTTGTCTAC
CATCATGTGACCAACGCGGATCTCTTCTGCACAAAAGAGGAGAATATTGGGA
GCATCGGTACAGTGGGTAGGAAGTGTAGTGTGAACAGCTACGGGCCTGATGC
TTGTGCCAACTTGTGTTGCAATCGAGGATTCATGGACAAGCCCCGAGTTGTAC
AGTTCGATTGCGATTGTAAATTCGCGTATGAACACTTGGACATGCGGTGTAAC
CAATGCCAAAAGACAATCCAAGAAGCATTCTGTATGTAG

PbWntx
ATGTATCAACTTTTATCAGTCTTTGTCTTGTNCTGTCCGACCCTTGGAGCTTCC
ATCACACAGACAGTGGCTACAGCGGGTGCTGCAGTGTTCGGAGTGAACACTG
AACAAACCATCAAGTACTACCACCGTGAAACCACAGAAAGTCCTCACGGCAC
GACGAGAAGCATGGGCTTGGCGACAGAGGCTTGTCAGATCTTTGAAGGGTTG
ACCCACCGGCAGAGGGCGGTGTGTGCTGAGGCCCCCGTCCTAATCCCTATCA
TGATTGAGGGAATAAACGGAGCAATCGAGACTTGCCGGGAAGCCTTCGGGTA
CCGTCGCTGGAACTGCAGTTCATTTGATATAGGGGTTGTGTTTGGGAAGAAG
ACGAAGCCCCAGCTCCCGGGAACAAAAGAGAGGGCAGTTATGCATGCCATG
GCGAGTGCCGGAGTTGTCATC

PbWnt9
CCAATCGCTCACTCTGCAGGAAGTTCGTGGCACATGAACGAGTGTAGTGATA
ACGTGGAAGTAGCATCTAAATTAGTGCTCGAACCTCCCACAACCCGATCACG
ACGGAACGTGGATACAAAAGCGCTGGTTGTGGAACACAACAACCGACTAGG
CATCCAGGCCCTTCAAAACACGCTTACACTGGAGTGTAAATGTCACGGCATA
AGTGGAACATGTGCAACTAAAACCTGCCTCCGAAAGATGCCCTCCTTCTCAC
TTATTGGCGACTACCTAAACCACAAATTCCATCTTGCTCGACGAGTCCTGCAC
AGCCGATCCGGGTATGAATTGAGAGTACCAGCAAAGGGAGGCCAGACACGA
GAACCTCGTCTAGATGAACTCTTGTACTACGAGAAAAGTCCCTCATTCTGCCT
GCCCGACCCGGACCTAGGTTGGGCGGGGACCTCTGGTCGAGAGTGTTCTCTC
CACTCGTCTGTCAGCATGTATGGTCGGAGTAGGGATAGCTGTGATGCGTTGTG
TTGTAATAGAGGATACTACACTAGGTATCAAACGGTAGAAGTGGACTGTCAT
TGTAGATATGTGCATTGTTGCGAGGTGAGGTGTGAGAGATGTAACACCACTC
AGCCC

P. bachei amino acid sequences used for alignments and analysis
Wnt x
MYQLLSVFVLXCPTLGASITQTVATAGAAVFGVNTEQTIKYYHRETTESPHGTTR
SMGLATEACQIFEGLTHRQRAVCAEAPVLIPIMIEGINGAIETCREAFGYRRWNCS
SFDIGVVFGKKTKPQLPGTKERAVMHAMASAGVVIAVARACQNGEVDKCGDCE
SGNVSAGASYQWGSCSNEIWNSAQFAKRMLDARENEYTSLSALNLHNNNVGRE
TIRESTAKNCRCHGPSSSCSIKTCWESLPSMSKIAKKLVTKYDIAMYAVSTRRRT
GQVKLRAREAADPERGRRSAATGDQITKLYYLKRSPSYCDADTSIGTPGTSGRN
CRLIAKKDG

Wnt 6
MRRYLKSIHVMDVGYRKWLLYDQNDFQPSPSDLVYHHVTNADLFCTKEENIGSI
GTVGRKCSVNSYGPDACANLCCNRGFMDKPRVVQFDCDCKFAYEHLDMRCNQ
CQKTIQEAFCM
Wnt 9
MKSNRCSKDIPIAHSAGSSWHMNECSDNVEVASKLVLEPPTTRSRRNVDTKALV
VEHNNRLGIQALQNTLTLECKCHGISGTCATKTCLRKMPSFSLIGDYLNHKFHLA
RRVLHSRSGYELRVPAKGGQTREPRLDELLYYEKSPSFCLPDPDLGWAGTSGRE
CSLHSSVSMYGRSRDSCDALCCNRGYYTRYQTVEVDCHCRYVHCCEVRCERCN
TTQPLHY

***In Situ* Hybridization** (Adapted from Derelle and Manuel 2007 and Moroz) :

Whole adult *P. bachei* were fixed in 4% paraformaldehyde in Filtered Seawater overnight (12 hours) at 4 ° C in a 50 ml conical tube. Animals were then rinsed three times for 10 minutes in phosphate buffered saline (PBS) with tween added (PTW) at room temperature. Then animals were washed in a 1:1 Methanol/ PTW for 10 minutes at room temperature. Animals were stored in 100% Methanol at -20° C in a 50 ml conical tube until needed.

Animals were rehydrated in 3:1, 1:1, 1:3, 0:1 ratios of Methanol and PTW for 10 minutes each at room temperature. Then washed in 1:1 solution of hybridization buffer for 15 minutes and then pre-hybridized for 1 hour in hybridization buffer at 60°C. This was followed by overnight incubation in 1 ml of hybridization buffer with the DIG-RNA probe.

Next animals washed in fresh hybridization buffer and, 1:1 Hybridization buffer/ PTW both for 30 minutes at 60°C , followed by a 30 minute wash in PTW at room temperature. Next samples were blocked in 10% goat serum for 1 hour at room temperature and incubated overnight in anti-DIG at a 1:2000 dilution in 1% goat serum overnight at 4°C.

The following day animals were washed 4 times at 30 minutes in phosphate buffered saline (PBS) at room temperature. Samples were then transferred from PBS to 24 well plates with 1ml detection buffer with 20 ul of NBT/BICP added.

Detection was allowed to continue on ice with foil covering the tray until signal strength, visualization and contrast is optimum. Samples were stopped in 4% paraformaldehyde in methanol at room temperature for 30 minutes followed by 10-minute washes in ethanol to remove any excess background development .

Mounting:

Adult *P. bachei* were mounted by dissecting under dissection microscope for proper visualization of area of expression. Samples were then placed in methyl salicylate and allowed to sink, samples were then transferred onto a microscope glass slide. Left over methyl salicylate was then removed and permount added over sample and coverslipped.

Phylogenetic and genomic analysis:

The *P. bachei genome* was scanned for sequences using the Moroz lab genome browser. Genes of interest were identified using a reciprocal Blast approach using *Wnt* pathway genes identified previously in *M. leidyi, Homo sapiens* and *A. queenslandica* with a e value cut off $1x10^{-5}$, sequences were identified as missing from the pathway if Blast searches did not receive any sequences with e-values bellow $1x10^{-5}$. Sequences used for phylogenetic and pathway comparisons were identified using NCBI blast searching and JGI databases. Alignments were done using MEGA 5[10] and alignments and gene trees were done using phylogeny.fr[11-15], conserved domain families from sequences were identified using SMART[16].

Results:

Initial search of the *P. bachei* genome indicates there are only 3 *Wnt* ligand genes in *P. bachei*, this is different than the 4 *Wnt* ligands genes in *M. leidyi* indicating that either *P. bachei* lost a *Wnt* from its genome or *M. leidyi* gained one after its divergence from their common ancestor. *Wnt* genes found in *P. bachei* are homologous to *Wnt X, Wnt 6* and *Wnt 9* in *M. leidyi*. *P. bachei* contains no homolog to the *M. leidyi Wnt A* gene (Figure 2). Many of the same components of the *Wnt* pathway that were present in *M. leidyi* are also present in the *P. bachei* genome including secreted frizzled receptor protein (sfrp), which is a Wnt antagonist that can bind to both the Wnt ligand and frizzled receptor. Also present in the *P. bachei* genome is the *frizzled* receptor and its co-receptor *LRP*, along with *dishevelled* which encodes a protein that interacts with the frizzled LRP complex. Other conserved pathway gene members present are *ß-catenin*, transcriptional factor *TCF/LEF* that interacts with the pygopus protein to create the transcriptional activation complex. Another part of the transcriptional activation complex is the histone acetylase, CREB binding protein (CBP), which relaxes the chromatin to further activate transcription, is also found in the genome. Parts of the destruction complex that are present include a dix domain protein which was a partial match to the dix domain in Axin and GSK-3. Missing from the *P. bachei* genome but present in the *M. leidyi* genome[8], is *APC* which encodes a protein that helps Axin and GSK-3 bind beta-catenin in the cytoplasm to aid in its destruction. Both ctenophores are missing common Wnt *antagonists Dikkopf, Wnt Inhibitory Factor and Cerberus* (Table 1).

Wnt domains normally contain conserved cysteine residues and many of the N-glycosylation sites[17] and like most secreted proteins have a transmembrane domain. All three sequences from *P. bachei* had a single identifiable Wnt domain (Figure 3). The predicted *Wnt x* gene is 335 amino acid in length and contains 15 of the 22 cysteine residues. The lack of cysteines could be due to evolutionary changes or the gene we have currently predicted may not be full length. *Wnt X* also contains a signal peptide at its 5'end, consistent with characteristics of a secreted ligand. The current predicted protein *Wnt 6* in *P. bachei* is 117 amino acids long and contains 11 cysteine residues. The current predicted protein *P. bachei Wnt 9 is* 223 amino acids long and contains 16 conserved cysteine residues (Figure 4). Because most Wnt proteins are between 350 and 400 amino acids long[18] I believe that further research and RACE PCR on these genes will need to be done in order to retain full length clones and that doing so will find *P. bachei Wnts* contain more of the 22 conserved cysteine residues.

PCR was performed on cDNA libraries of embryos ranging from 1 cell through 1 day, in order to investigate the presence of *Wnt* in development, and visualization showed expression of *Wnt 6-Wnt 9* in all stages in *P. bachei*. However *Wnt X* did not show expression in any stage except the 1 day (Figure 5A). *In Situ* hybridization in *P. bachei* with the *Wnt X* probe showed expression at many different stages of development (Figure 5B). This conflicted with our PCR results, two possibilities could explain this result either the *Wnt* probes could be cross-reacting or the PCR reaction simply did not work both experiments need to be repeated in future research.

mRNA expression studies were done on adult *P. bachei* using *In situ* hybridization using portions cloned from all 3 *Wnt* sequences as probes. *Wnt X* showed expression in the ciliated groove, polar fields, the filaments and bulbs of the tentacles and comb rows (Figure 6A-C). *Wnt 6* also had expression in the comb rows, tentacle filaments and additionally had expression in the mouth (Figure 7). *Wnt 9* had light expression in the comb rows and distinct expression in the tentacle filaments (Figure 8).

Discussion:

Though most of the canonical Wnt pathway components are present in basal metazoans such as ctenophores, it appears that there was a rapid expansion of the pathway (Figure 9) prior to the divergence of cnidarians. Specifically, the *Nematostella vectensis* genome contains 11 *Wnt* ligands and the antagonists *Dickkopf* and *WIF* [3,19]. This indicates a large expansion in the early metazoan lineage, which caused the *Wnt* pathway to become more complex and modulated by additional regulators to be involved in numerous complex processes.

Also interesting is the loss of antagonists in many lineages after the cnidarian lineage, as *Dickkopf* was lost in the members of Ecdysozoa, *C. elegans* and *D. melanogaster*, with *C. elegans* having an additional lost of *WIF*[20,21], These lack of antagonists in basal metazoans and losses in other lineages supports the idea that these antagonists are not necessary for Wnt pathway function, but asks the question, "why did they evolve and does the pathway function the same way in organisms with and without these antagonists?" Though the antagonist to Wnt, *secreted frizzled receptor protein (sfrp)* is present in lineages missing other antagonists it still does not answer the question of how lacking these antagonists effects the pathway functions. Missing members of the destruction complex in basal metazoans also indicate that all members of the beta-catenin destruction complex may not be necessary for proper pathway function. Additional components may have been added later to refine the pathway and expand its function. We are interested in what process the canonical Wnt pathway originally evolved for due to the missing antagonists and other components of the pathway.

Embryonic *Wnt* expression results indicate that the pathway plays a role in *P. bachei* development. Previously reported studies in *Mnemiopsis leidyi* didn't detect *Wnt* expression until late in development after gastrulation[9]. This indicates either the *Wnt* pathway is functioning differently in the different ctenophore species or the different in *situ* hybridization protocols could have produced different results. In order to resolve this question results need to be repeated and reproduced.

The *in situ* hybridization of *Wnt X* expression was localized and most concentrated in the 8 ciliated grooves, each of which innervate and connect, one comb row to the apical organ which functions as the sense organ and plays a role in balance and orientation, Polar fields are presumed to have a sensory function, and comb rows are used for movement. Tentacles are used for the gathering of food as the tentacles contract and bring food to the mouth.[2] This expression pattern indicates that in adult *P. bachei Wnt X* is important for maintaining patterning and function of the sense organs. The expression pattern in *Wnt 6* and *Wnt 9* in the combs and tentacle filaments indicate they may play a similar functional role. All of the structures where *Wnt* expression was localized are structures for coordinated movement. Recent research has shown that *P. bachei* is missing many of the conserved neurotransmitters (Moroz, unpublished). Due to this lack of classical neurotransmitters and the localization of *Wnt* in the adult *P. bachei, Wnt,* which is a secreted ligand, could be playing a role in neurotransmission in the adult.

Future directions:

Current research has yet to determine what role Wnt pathway had initially evolved for. Additional research and experiments to confirm the *Wnt* pathway is functional in *P. bachei* could be done by antibody detection of nuclear beta-catenin. Additional studies such as inhibition and over-expression of the *Wnt* pathway in *P. bachei* embryos could show if *Wnt* is playing a similar role in development in P. bachei. Adult treatment with *Wnt* specific inhibitors for detection of effects on behavior is needed in order to further investigate the role of *Wnt* as a neurotransmitter. In addition *Wnt* expression should be studied in adults of other species in order to see if this high adult expression of *Wnt* is specific to *P. bachei* or common to the metazoan clade.

Acknowledgments:

I would like to than my professors Dr. Billie Swalla, Dr. Leonid Moroz and Dr. Andrea Kohn for advising me and helping me along the way of this project. I would also like to thank the Marine Genomics TAs, Caleb Bostwick , Emily Dabe and Gariella Winters and Mat Citarella, and all my fellow classmates from the Marine Genomics Apprenticeship for helping with my project throughout the quarter. Thank you to the Mary Gates Endowment, NIH and NSF for funding this work.

References

1 Mills, C. E. *Phylum Ctenophora: List of all valid species names*, 2012).

2 Brusca, R. C. & Brusca, G. J. *Invertebrates*. (Sinauer Associates, 1990).

3 Kusserow, A. *et al.* Unexpected complexity of the Wnt gene family in a sea anemone. *Nature* **433**, 156-160, doi:10.1038/nature03158 (2005).

4 Srivastava, M. *et al.* The Amphimedon queenslandica genome and the evolution of animal complexity. *Nature* **466**, 720-726, doi:10.1038/nature09201 (2010).

5 Church, V. L. & Francis-West, P. Wnt signalling during limb development. *The International journal of developmental biology* **46**, 927-936 (2002).

6 Lee, P. N., Pang, K., Matus, D. Q. & Martindale, M. Q. A WNT of things to come: evolution of Wnt signaling and polarity in cnidarians. *Seminars in cell & developmental biology* **17**, 157-167, doi:10.1016/j.semcdb.2006.05.002 (2006).

7 Ryan, J. F. & Baxevanis, A. D. Hox, Wnt, and the evolution of the primary body axis: insights from the early-divergent phyla. *Biology direct* **2**, 37, doi:10.1186/1745-6150-2-37 (2007).

8 Katoh, M. & Katoh, M. WNT signaling pathway and stem cell signaling network. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 4042-4045, doi:10.1158/1078-0432.CCR-06-2316 (2007).

9 Pang, K., Ryan, J. F., Mullikin, J. C., Baxevanis, A. D. & Martindale, M. Q. Genomic insights into Wnt signaling in an early diverging metazoan, the ctenophore Mnemiopsis leidyi. *EvoDevo* **1**, 10, doi:10.1186/2041-9139-1-10 (2010).

10 Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**, 2731-2739, doi:10.1093/molbev/msr121 (2011).

11 Anisimova, M. & Gascuel, O. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Systematic biology* **55**, 539-552, doi:10.1080/10635150600755453 (2006).

12 Chevenet, F., Brun, C., Banuls, A. L., Jacq, B. & Christen, R. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC bioinformatics* **7**, 439, doi:10.1186/1471-2105-7-439 (2006).

13 Dereeper, A., Audic, S., Claverie, J. M. & Blanc, G. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol Biol* **10**, 8, doi:10.1186/1471-2148-10-8 (2010).

14 Dereeper, A. *et al.* Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic acids research* **36**, W465-469, doi:10.1093/nar/gkn180 (2008).

15 DeSalle, R. & Schierwater, B. An even "newer" animal phylogeny. *BioEssays : news and reviews in molecular, cellular and developmental biology* **30**, 1043-1047, doi:10.1002/bies.20842 (2008).

16 Letunic, I., Doerks, T. & Bork, P. SMART 7: recent updates to the protein domain annotation resource. *Nucleic acids research* **40**, D302-305, doi:10.1093/nar/gkr931 (2012).
17 Mikels, A. J. & Nusse, R. Wnts as ligands: processing, secretion and reception. *Oncogene* **25**, 7461-7468, doi:10.1038/sj.onc.1210053 (2006).
18 MacDonald, B. T., Tamai, K. & He, X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Developmental cell* **17**, 9-26, doi:10.1016/j.devcel.2009.06.016 (2009).
19 Guder, C. *et al.* The Wnt code: cnidarians signal the way. *Oncogene* **25**, 7450-7460, doi:10.1038/sj.onc.1210052 (2006).
20 McQuilton, P., St Pierre, S. E. & Thurmond, J. FlyBase 101--the basics of navigating FlyBase. *Nucleic acids research* **40**, D706-714, doi:10.1093/nar/gkr1030 (2012).
21 Todd W. Harris, I. A., Tamberlyn Bieri, Darin Blasiar, Juancarlos Chan, Wen J. Chen, Norie De La Cruz, Paul Davis, Margaret Duesbury, Ruihua Fang, Jolene Fernandes, Michael Han, Ranjana Kishore, Raymond Lee, Hans-Michael Müller, Cecilia Nakamura, Philip Ozersky, Andrei Petcherski, Arun Rangarajan, Anthony Rogers, Gary Schindelman, Erich M. Schwarz, Mary Ann Tuli, Kimberly Van Auken, Daniel Wang, Xiaodong Wang, Gary Williams, Karen Yook, Richard Durbin, Lincoln D. Stein, John Spieth and Paul W. Sternberg. *Wormbase:facilitating insights into nematode biology*, <http://www.wormbase.org> (2012).

Gene	*P. bachei*	*M. leidyi*	*A. queenslandica*
Wnt	3	4	3
Frizzled (Fzd)	2	2	2
Secreted-frizzled related protein (Srfp)	Present	Present	Present
LRP	Present	Present	Present
APC	No?	Partial Present	Missing domains
Axin	Dix-domain like protein	Dix-domain like protein	Axin domain with no ß-Catenin binding domain
GSK3	Present	Present	Present
Dishevelled	Present	Present	Present
ß-catenin	Present	Present	Present
TCF/LEF	Present	Present	Present
CK1	Present	Present	Present
groucho	Present	Present	Present
WIF	Absent	Absent	Absent
Dickkopf	Absent	Absent	Absent
Cerberus	Absent	Absent	Absent
CREB-biding Protein (CBP)	Present	Present	Present

Table 1: The canonical *Wnt* pathway components conserved in basal metazoans

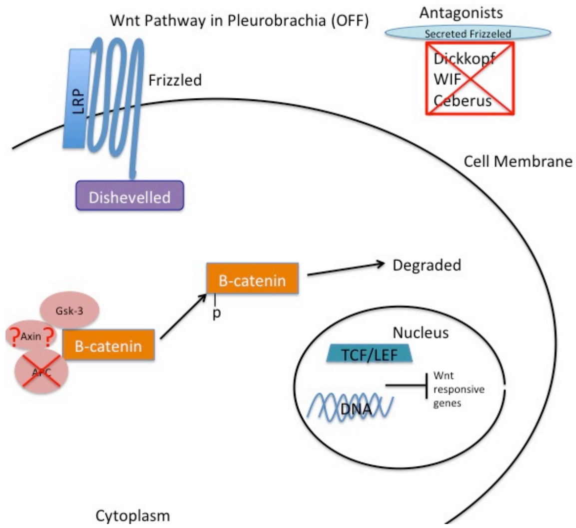


Figure 1 A: Conservation of the inactive *Wnt* Pathway in *P. bachei*
Wnt pathway in *P. bachei*. When the Wnt pathway is inactive the destruction complex of axin, adenomatous polyposis coli (APC) and gycogen synthase kinase 3 (GSK-3), phosphorylate ß-catenin and lead it its degradation. *Pleurobrachia bachei* genome appears to be missing several key components of the pathway including parts of the destruction complex and known antagonists that prevent wnt ligand binding with the frizzled receptor, Dickkopf, wnt inhibitory factor (WIF) and Ceberus.

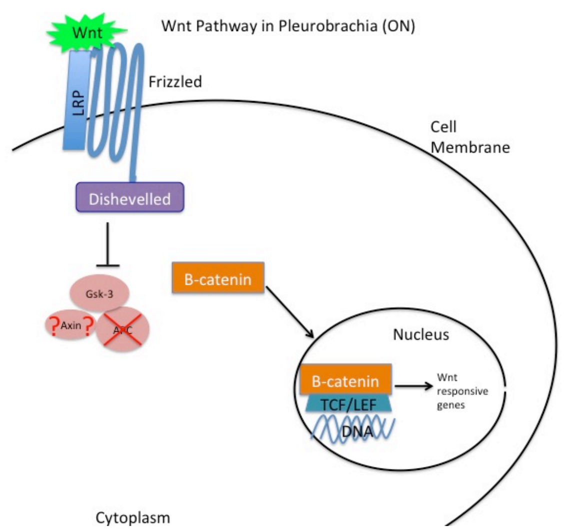


Figure 1 B: Conservation of the active *Wnt* Pathway in *P. bachei*
When the *Wnt* pathway is on, Wnt binds to Frizzled and its co-receptor lipoprotein receptor- related protein (LRP) this causes activation of Dishevelled. Dishevelled inhibits GSK-3. This allows ß-catenin to be released from the destruction complex. Once it is released it can enter the nucleus and interact with T-cell specific transcription factor/lymphoid enhancer binding factor (TCF/LEF). This then turns on Wnt responsive genes for transcription. In *P. bachei* the APC part of the destruction complex is missing from the genome. Also, only a partial match to the dix domain in axin was found. All other components in this diagram are present.

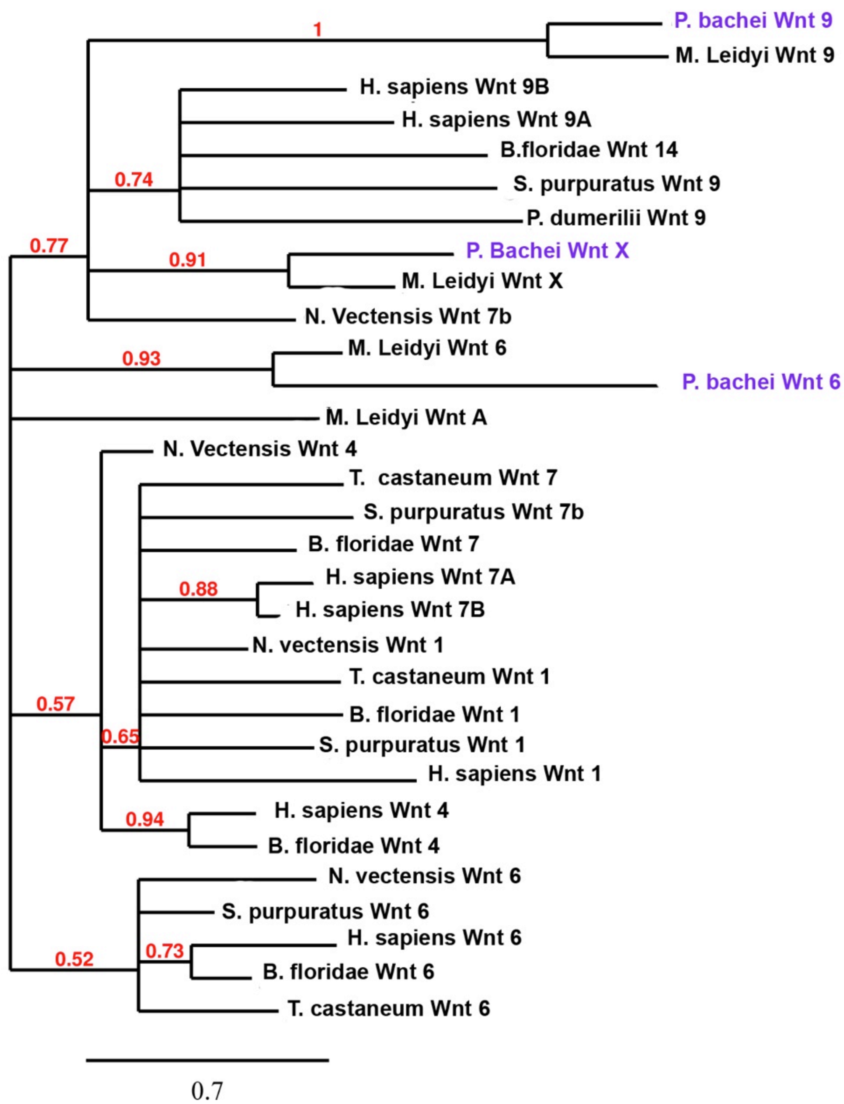


Figure 2: Conservation of *Wnt* genes between ctenophores
Phylogenetic analyses group ctenophore *Wnt* genes with high support. *Pleurobrachia bachei* is missing a gene complement to the M. leidyi *Wnt A*
Maximum likelihood analysis of *P. bachei Wnt* genes with bootstrap support indicated at each node and all nodes with support under 0.50 were collapsed. P. bachi= *pleurobrachia bachei;* M. leidyi= *Mnemiopsis leidyi;* H. sapiens= *Homo sapiens;* B. floridae= *Brachiostoma floridae;* S. purpuratus= strongylocentrotus purpuratus,; P. dumerilii= *Platynereis dumerilii;* N. vetensis= *Nematostella vectensis ;* T. castaneum = *Tribolium castaneum*

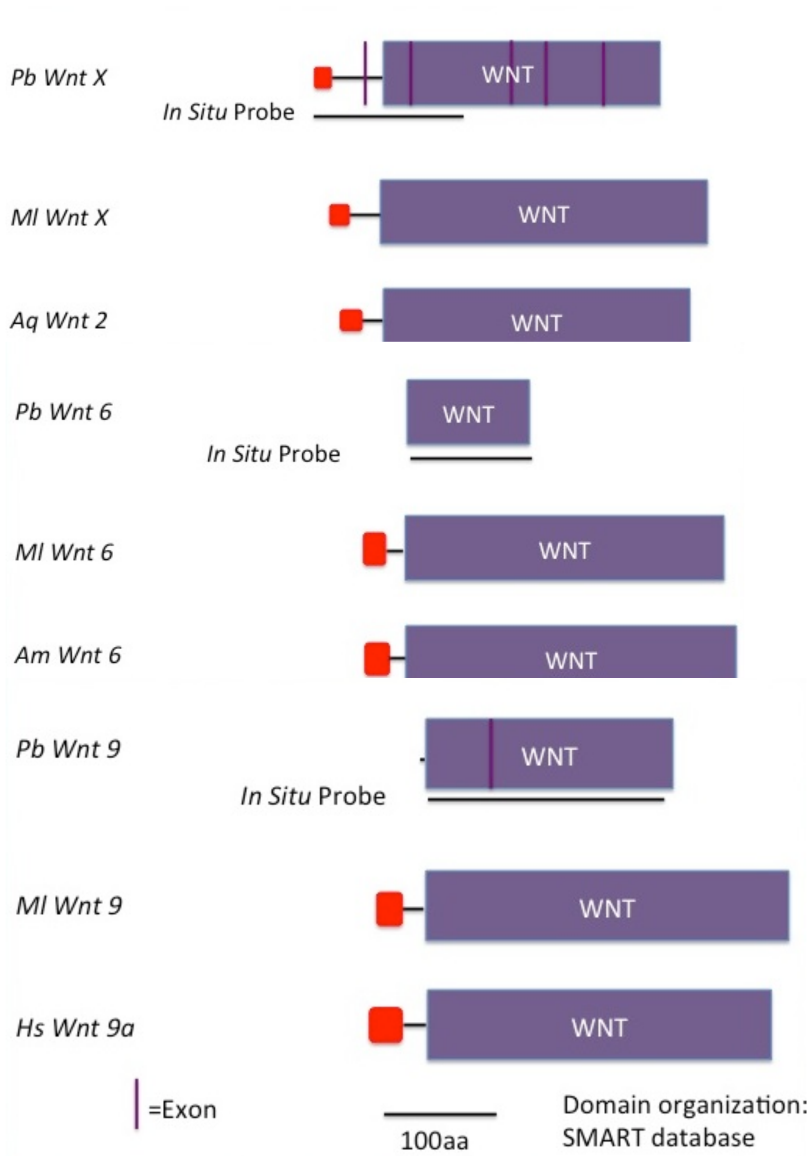


Figure 3: Conserved *Wnt* domains present in *P. bachei Wnt* genes
Domain organization, the conserved *wnt* pfam domain is shown in purple, dark purple lines indicate the exons in the *P. bachei Wnt* genes, red boxes indicate signal peptide domains and *in situ* probe location is indicated below the domain organization. With each *P. bachei Wnt* gene is the domain organization of *Wnt* genes from other species. *Pb= pleurobrachia bachei* Ml= *Mnemiopsis Leidyi;* Hs= *Homo sapiens;* Aq= *Amphemidon queenslandica*: Am= *Apis mellifera*

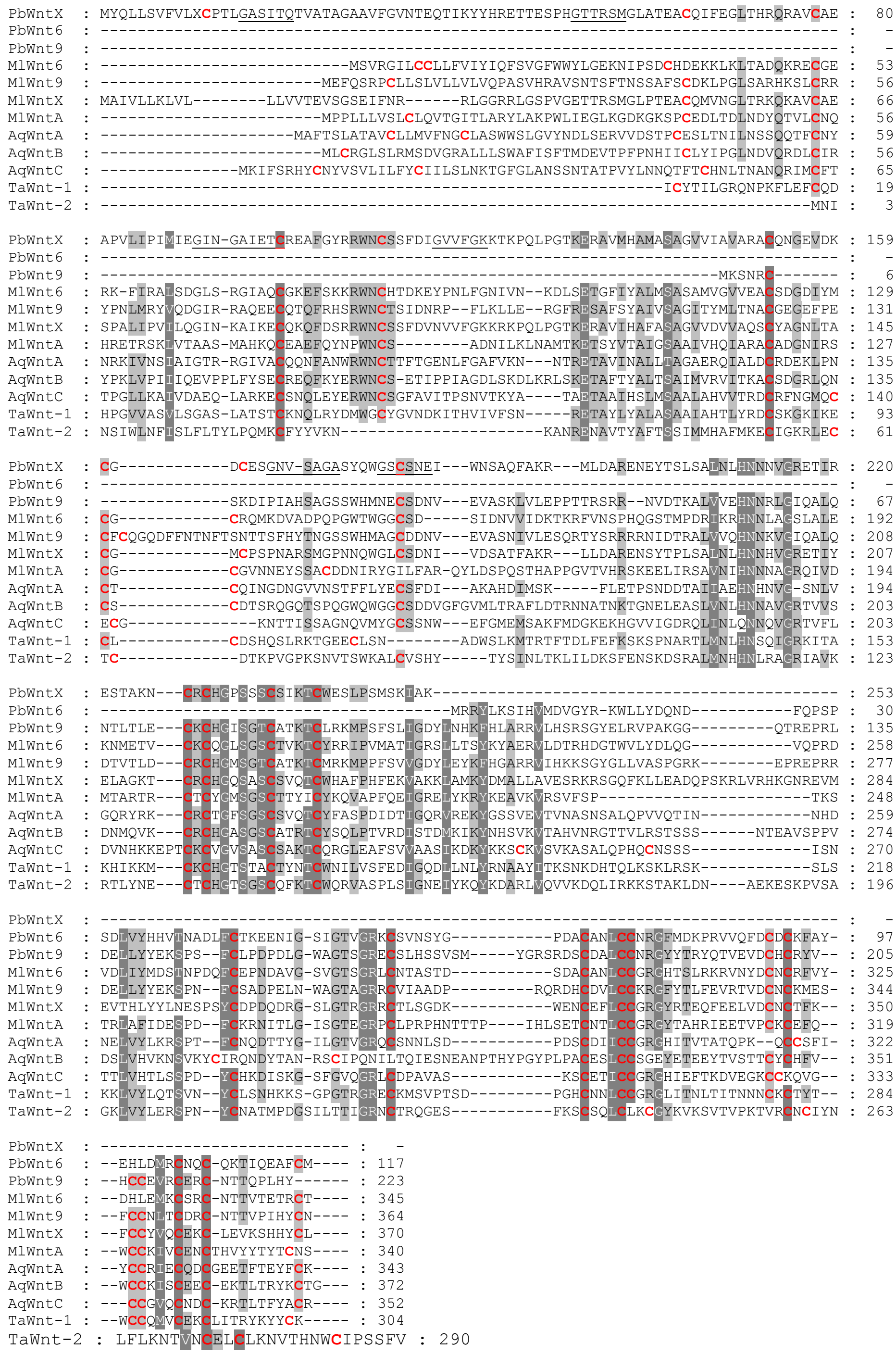


Figure 4: *P. bachei Wnts* contain conserved cysteine residues and N-myristoylation sites (by Andrea Kohn) Alignments show that *P. bachei Wnts* contain many of the conserved cysteine residues shown in red and N-myristoylation sites underlined in black. Pb= *Pleurobrachia bachei* Ml= *Mnemiopsis leidyi* and Ta= *Trichoplax adhaerens*

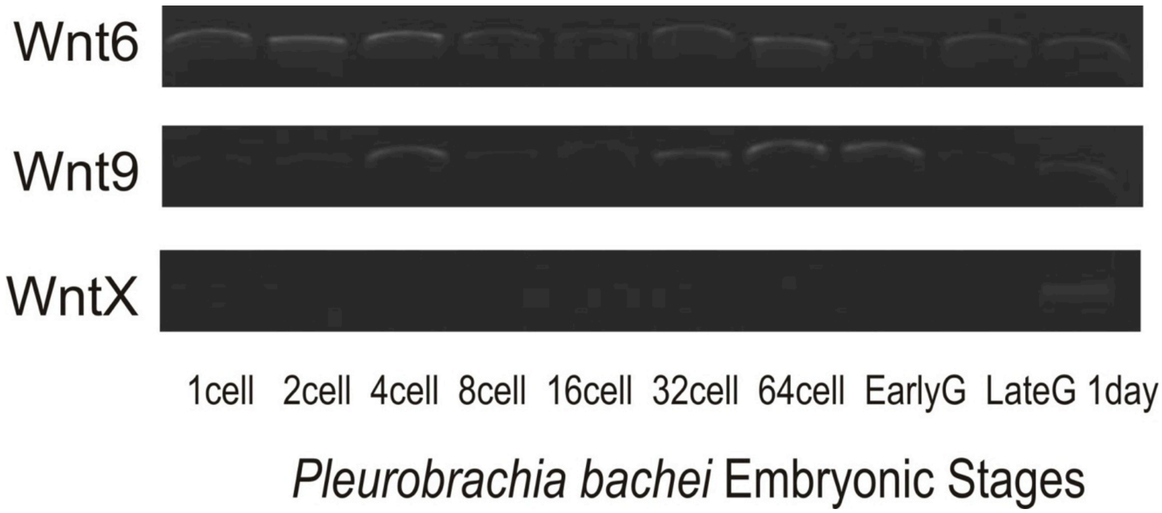


Figure 5A: Embryonic Expression of *P. bachei Wnt* genes
PCR using primers specific for each of the *Wnt* genes PCR was performed on cDNA libraries for embryonic stages 1 cell - 1 day results indicated expression results for *Wnt 6* and *Wnt 9* genes. *Wnt X* only showed expression in the 1 day stage library indicating is expression beings some time after gastrulation. Results need to be repeated because this conflicts with the *Wnt X* expression

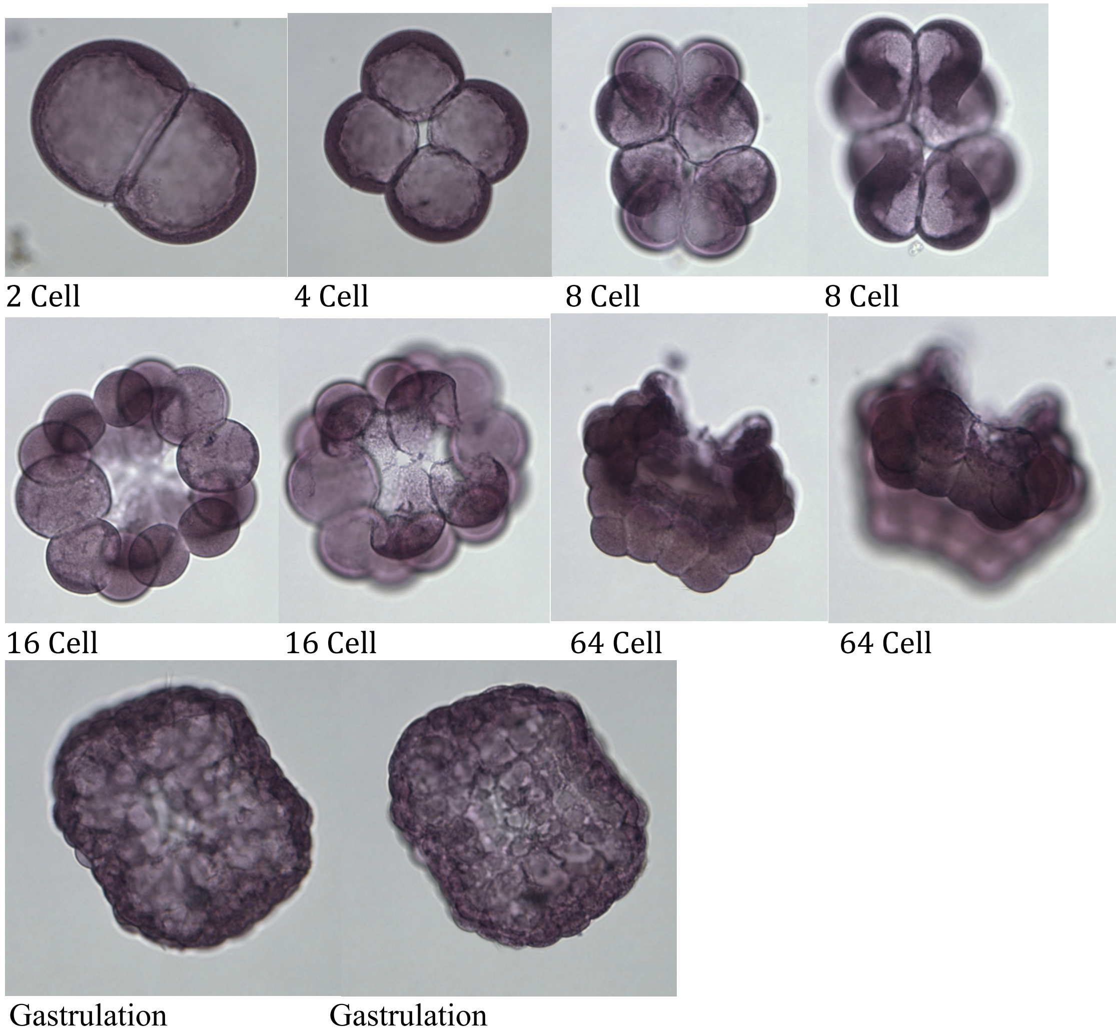


Figure 5B: Embryonic *Wnt* expression
in situ hybridization shows *Wnt X* expression throughout many stages of development including 2 cell, 4 cell, 8 cell, 16 cell, 64 cell and gastrulation

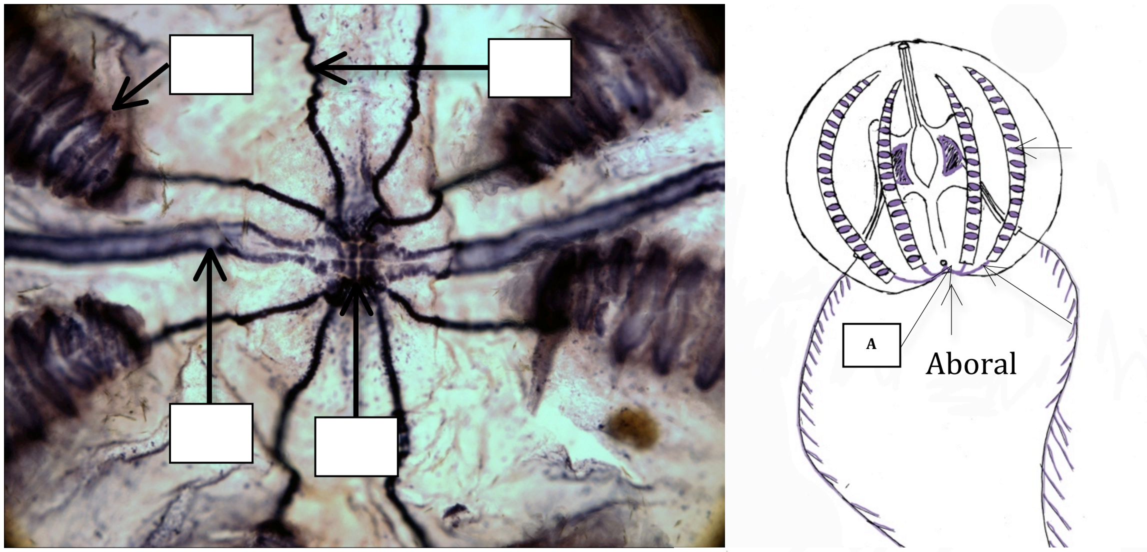
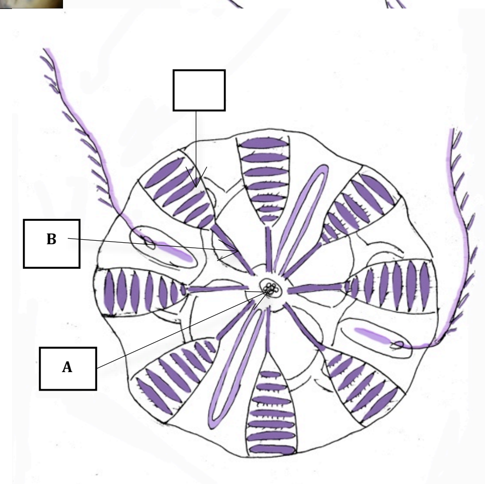


Figure 6 A: Aboral view of *Wnt X* expression
Aboral view of *Wnt X in situ* hybridization shows expression in the A. apical organ, B. Polar fields C. Comb rows and D. ciliated grooves



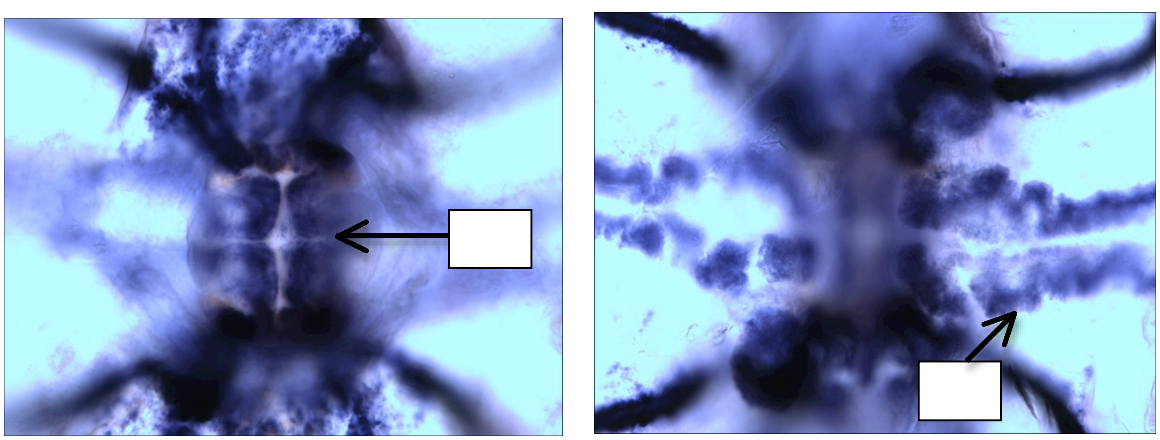


Figure 6B: aboral view of *Wnt X* expression
Aboral view of *Wnt X in situ* hybridization shows localized expression in A. Apical organ B. Polar fields

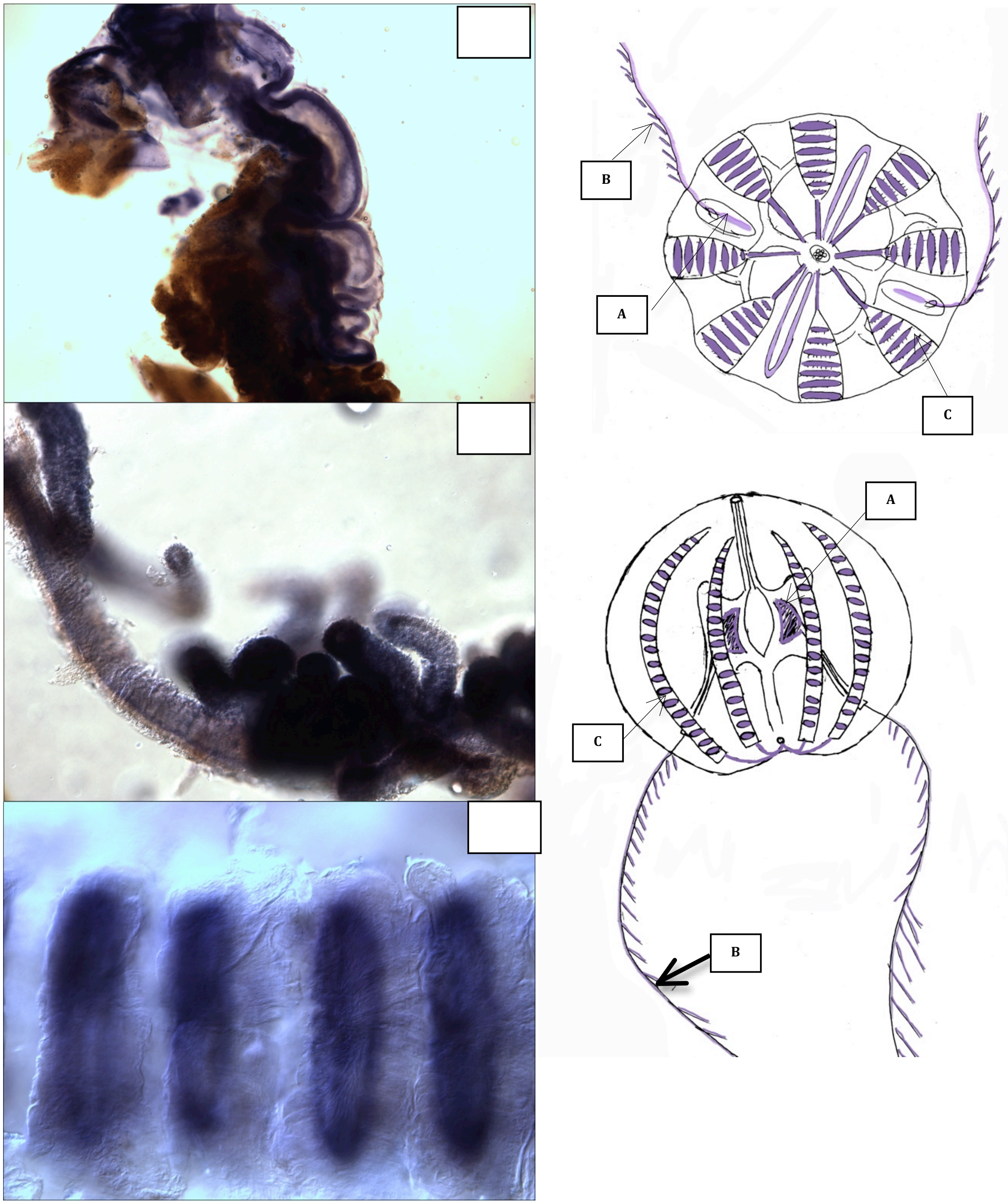


Figure 6 C: *Wnt X expression in combs and tentacles*
Wnt X in situ hybridization shows localized mRNA expression in A. the tentacle bulb B. the tentacle filaments and tentillae C. the combs

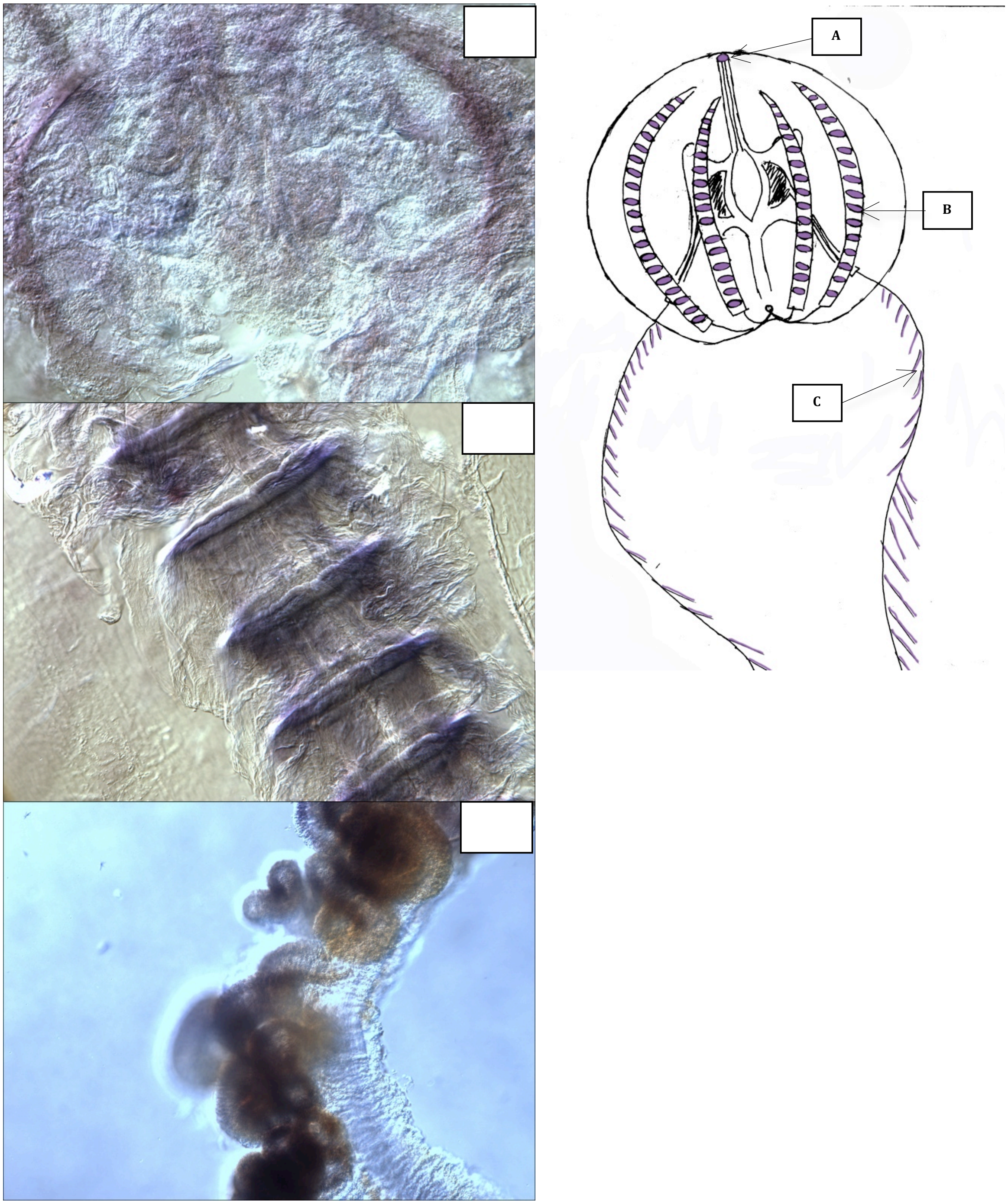


Figure 7: ***Wnt 6*** **expression**
Wnt 6 in situ hybridization showed localized expression of mRNA in A. the mouth B. the combs and C. The tentacle/tentillae

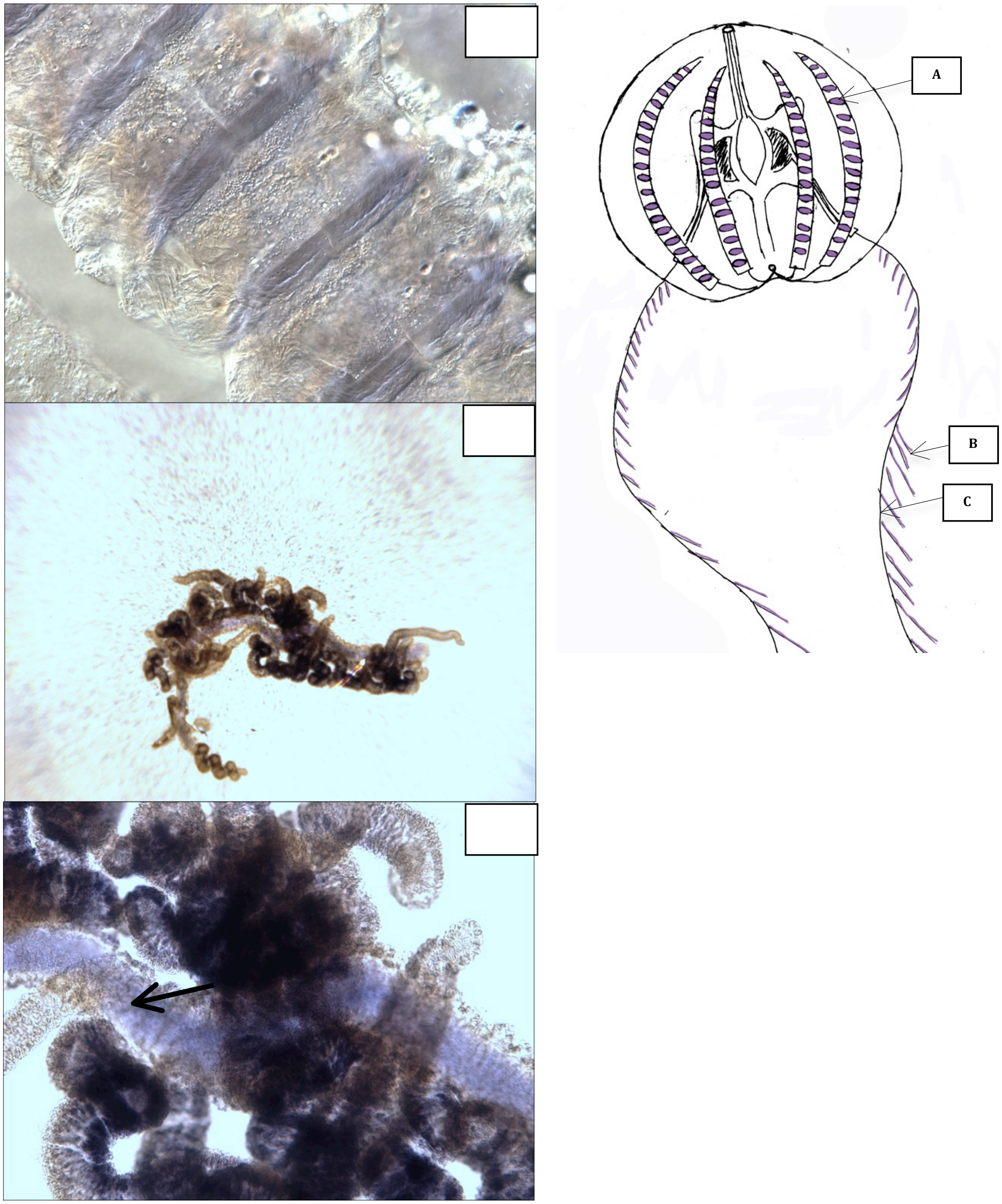
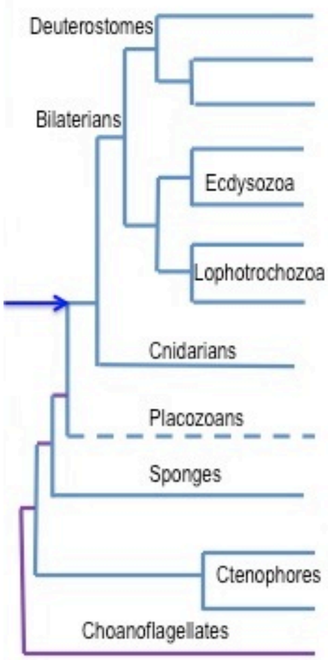


Figure 8: *Wnt 9* expression
Wnt 9 in situ hybridization showed localized expression of mRNA in A. The combs B. the tentillae C. in a structure running down the center of the tentacle indicated with an arrow



	Wnt	*Frizzled*	*Dickkopf*	*WIF*	*Ceberus*
Homo	19	Yes	Yes	Yes	Yes
Saccoglossus					
Branchiostoma	10+?	Yes	Yes	No	No
Drosophila	7	Yes	No	Yes	No
Caenorhabditis	5	4	No	No	No
Capitella	12	Yes	No?	Yes	No?
Lottia gigantea	11	Yes	No?	Yes	No?
Nematostella	11	Yes	Yes	Yes	No
Trichoplax	3	Yes	No	No	No
Amphimedon	3	Yes	No	No	No
Pleurobrachia	**3**	**Yes**	**No**	**No**	**No**
Mnemiopsis	4	Yes	No	No	No
Monosiga	0	No	No	No	No

Figure 9: Expansion of the *Wnt* pathway through the Opisthokonta
Very few *Wnt* ligand genes and antagonists are present in the basal metazoans and choanoflagelates, rapid expansion of these genes occurs prior to the divergence of cnidarians indicated by the blue arrow, with dramatic loss in the Ecdysozoa.