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Supplementary Information accompanies the paper on **www.nature.com/nature**

Acknowledgements We thank S. Emmons, Y. Fofanov, D. Graur, D. Portman, T. Shin, S. Srinivasan and M. Travisano for discussions. Z. Altun and D. Hall gave advice on the classification of *C. elegans* cells. The Sun Microsystems Center of Excellence in the Geosciences at the University of Houston provided access to high-performance computing resources. The Foundation for Science and Technology (Portugal), European Molecular Biology Organization, Biotechnology and Biological Sciences Research Council (UK), and the University of Houston provided financial support.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to R.B.R.A. (razevedo@uh.edu).

Unexpected complexity of the *Wnt* gene family in a sea anemone

Arne Kusserow[1], Kevin Pang[2], Carsten Sturm[1], Martina Hrouda[3], Jan Lentfer[1], Heiko A. Schmidt[4], Ulrich Technau[1]*, Arndt von Haeseler[4,5], Bert Hobmayer[3], Mark Q. Martindale[2] & Thomas W. Holstein[1,6]

[1]*Institute of Zoology, Darmstadt University of Technology, Schnittspahnstrasse 10, D-64287 Darmstadt, Germany*
[2]*Kewalo Marine Lab PBRC, University of Hawaii, 41 Ahui Street, Hawaii 96813 Honolulu, USA*
[3]*Institute of Zoology and Limnology, Center of Molecular Biosciences Innsbruck, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria*
[4]*John von Neumann-Institut für Computing (NIC), FZ Jülich, D-52425 Jülich, Germany*
[5]*Institut für Bioinformatik, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1 / Geb. 25.13.02, D-40225 Düsseldorf, Germany*
[6]*Department of Molecular Evolution and Genomics, University of Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany*

* Present address: Sars Centre, Thormøhlensgt. 55, N-5008 Bergen, Norway

The *Wnt* gene family encodes secreted signalling molecules that control cell fate in animal development and human diseases[1]. Despite its significance, the evolution of this metazoan-specific protein family is unclear. In vertebrates, twelve Wnt subfamilies were defined, of which only six have counterparts in Ecdysozoa (for example, *Drosophila* and *Caenorhabditis*)[2]. Here, we report the isolation of twelve *Wnt* genes from the sea anemone *Nematostella vectensis*[3], a species representing the basal group[4] within cnidarians. Cnidarians are diploblastic animals and the sister-group to bilaterian metazoans[5]. Phylogenetic analyses of *N. vectensis Wnt* genes reveal a thus far unpredicted ancestral diversity within the Wnt family[2,6,7]. Cnidarians and bilaterians have at least eleven of the twelve known *Wnt* gene subfamilies in common; five subfamilies appear to be lost in the protostome lineage. Expression patterns of *Wnt* genes during *N. vectensis* embryogenesis indicate distinct roles of Wnts in gastrulation, resulting in serial overlapping expression domains along the primary axis of the planula larva. This unexpectedly complex inventory of Wnt family signalling factors evolved in early multicellular animals about 650 million years (Myr) ago, predating the Cambrian explosion by at least 100 Myr (refs 5, 8). It emphasizes the crucial function of *Wnt* genes in the diversification of eumetazoan body plans[9].

We isolated twelve *Wnt* genes from *N. vectensis*, yet only one orthologue (*Wnt3*) was identified from the freshwater polyp *Hydra magnipapillata*[6]. Alignments of these cnidarian sequences were made using representatives in known databases from all three major metazoan clades: that is, deuterostomes (including all human sequences), ecdysozoans, and lophotrochozoans (Supplementary Tables S1 and S2). Phylogenetic analyses were based on three different phylogenetic methods: that is, the maximum parsimony (MP) and maximum likelihood (ML, TREE-PUZZLE and IQPNNI) approaches (Supplementary Figs S1–S3) and bayesian phylogenetic inference (Fig. 1). All approaches generated twelve *Wnt* gene subfamilies identified as *WntA* and *Wnt1–11*. Cnidarians possess orthologues of eleven of the twelve *Wnt* subfamilies, *WntA*, *Wnt1–8,* and *Wnt10–11* (Table 1). Only *Wnt9* was not found in cnidarians. It remains unclear whether we failed to identify this gene in *N. vectensis* or whether *Wnt9* has been lost in cnidarian evolution. The sea anemone *NvWnt* subfamilies *NvWnt7* and *NvWnt8* exhibit two paralogous genes which share no orthology with the same *Wnt* subfamilies in mammalians (Fig. 1). Therefore, they represent cnidarian or anthozoan specific duplications.

Thus at least eleven of twelve *Wnt* gene subfamilies must have already been present before the divergence of bilaterians and cnidarians. They constituted the *Wnt* repertoire of the last common ancestor of bilaterians and cnidarians, the *Ur-Eumetazoa* (see Table 1). Our comparison also indicates the existence of only seven *Wnt* gene subfamilies (*WntA, -1*, *-5–7* and *-9–10*) in insects and only five *Wnt* genes in *Caenorhabditis elegans* (Table 1). Full genome sequences are available from these three species (*C. elegans*, *Drosophila melanogaster* and *Anopheles gambiae)* so it is highly unlikely that we missed *Wnt* orthologues from ecdysozoans in our analysis. In lophotrochozoans, the second major protostomian clade, *Wnt* gene subfamilies *Wnt3*, *-6*, *-8*, and *-11* have not been reported yet[2,10]. Thus it remains to be clarified which *Wnt* gene subfamilies existed at the protostome–deuterostome divergence. In turn, our data reveal that only one *Wnt* gene subfamily (*WntA*) was lost during the evolution of deuterostomes (Table 1).

Although the *Wnt* gene subfamilies are statistically well supported, there is not enough phylogenetic resolution to distinguish reliable relationships among all *Wnt* subfamilies. Nonetheless, there is a clustering of the *Wnt1*, *-6*, *-10*, *-9* and *-3* subfamilies in the phylogenetic data (Fig. 1), which is also supported by human and fly genome data[11]. In the *D. melanogaster* genome, *DmWnt1* (*Wg*), *DmWnt6* and *DmWnt10* are positioned immediately adjacent to each other on the second chromosome and transcribed in the same orientation. This order is conserved in the mammalian genome, where also *Wnt3A* and *-9A* and *Wnt3* and *-9B* are closely linked[11]. Thus, *Wnt* genes *Wnt1*, *-6*, *-10*, *-9* and *-3* might represent an ancestral cluster of *Wnt* genes that originated in the evolution of the common ancestor of cnidarians and bilaterians. No *Wnt* genes have been described so far from unicellular eukaryotes, from cellular slime moulds (*Dictyostelium discoideum*) or from choanoflagellates[12], unicellular and colonial Protozoa that are closely related to Metazoa. At present no data are available from sponges, which probably diverged before the origin of the eumetazoan ancestor, but we presume that the appearance of *Wnt* genes itself was linked to the origin and evolution of multi-cellular animals from single-cell (protozoan) ancestors.

To analyse the possible function of different *Wnt* genes in *N. vectensis* embryogenesis, *Wnt* gene expression for ten genes was assayed by *in situ* hybridization from the early blastula through to newly settled polyps forming their first tentacles (Fig. 2). Each *Wnt* gene displayed a distinct expression pattern during early embryogenesis. Most of the *N. vectensis Wnt* genes are expressed along the primary body axis, where they are restricted to the blastopore during gastrulation and to the oral region of planula or polyps

(*NvWntA*, *-1–2*, and *-4–8*). Each subfamily of *Wnt* genes is also restricted to one of the two body layers, the ectoderm (Fig. 2a–y) or the endoderm (Fig. 2a′–o′). Except for *NvWnt11* (see below) no *Wnt* gene expression was detected by reverse-transcription polymerase chain reaction (RT–PCR) during the early cleavage stages (data not shown).

Five *Wnt* genes (*NvWntA*, *-1–2*, *-4* and *-7*) are expressed in staggered domains in the ectoderm, and together they span the

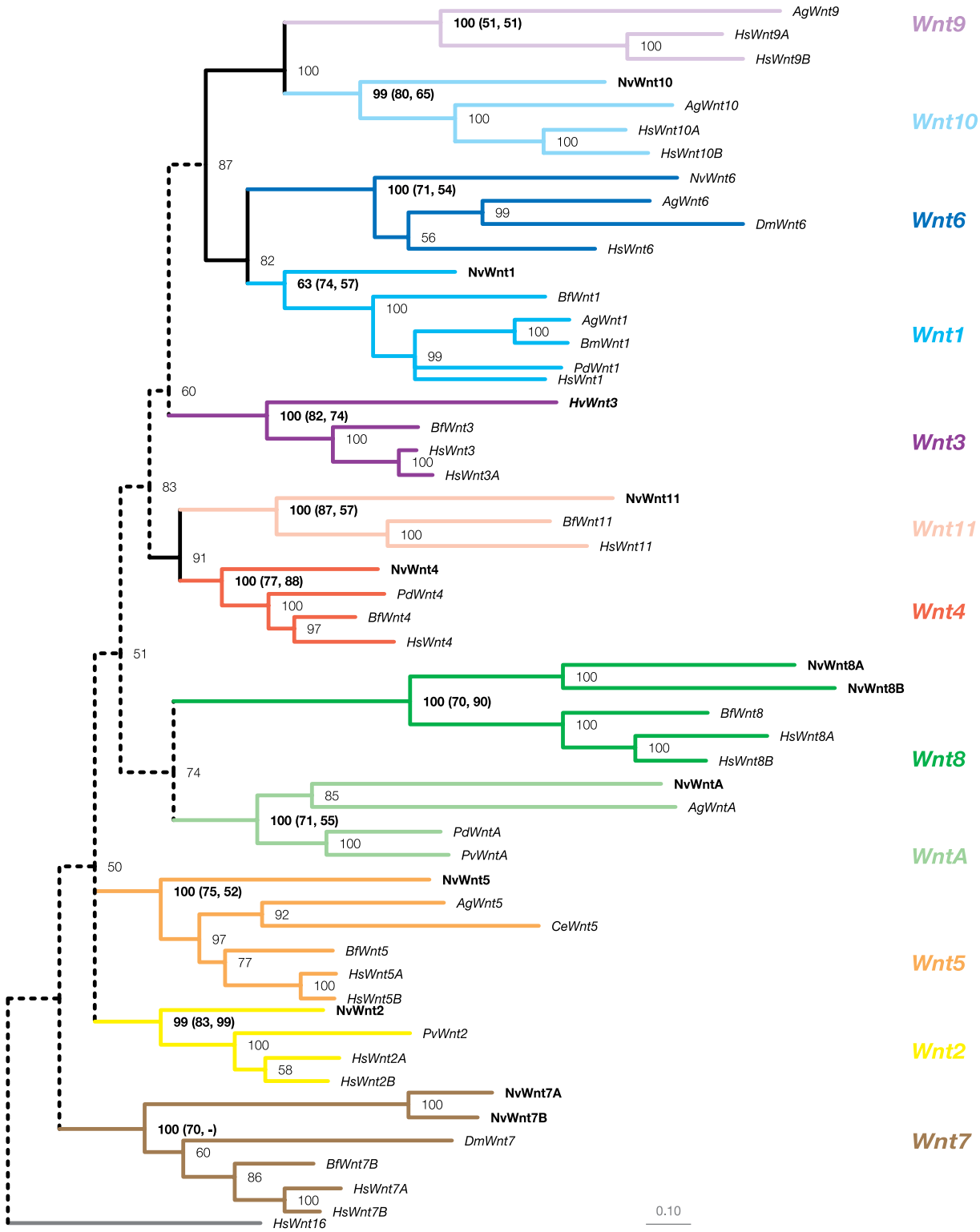

Figure 1 Bayesian inference consensus tree of the *Wnt* gene family. Numbers right of branches represent support values from different analyses. Bayesian support values are given on all branches, support values found by MP (Paup) and ML (TREE-PUZZLE) approach are in brackets (bootstrap values and quartet puzzling support). Species abbreviations: *Bf*, *Branchiostoma floridae* (amphioxus); *Bm*, *Bombyx moori* (insect); *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Hs*, *Homo sapiens*; *Hv*, *Hydra vulgaris*; *Nv*, *Nematostella vectensis* (sea anemone); *Pd*, *Platynereis dumerlii* (polychaete); *Pv*, *Patella vulgata* (mollusc), *Ag*, *Anopheles gambiae*. Bilaterian genes are italicized, *N. vectensis* genes are in bold, the *H. vulgaris* gene is italic and bold.

entire oral–aboral axis except for the aboral pole itself (Fig. 2). *NvWntA* expression commences in the early gastrula as a broad expression domain defining the site of gastrulation and extends into the entire involuting ectodermal epithelial layer at late gastrula stages (Fig. 2b, Supplementary Fig. S4). *NvWnt1*, *-2* and *-4* are expressed around the blastopore at the start of gastrulation with *NvWnt1* expressed at the most oral extremity, *NvWnt4* a bit more aboral, and *NvWnt2* forming a large stripe in the middle of the embryo (Fig. 2). *NvWnt7* expression is also restricted to the oral end of planula and polyp, similar to *NvWnt1*, but its expression does not start before gastrulation is completed (Fig. 2u–y, Supplementary Fig. S5). A similar distribution of gene expression is seen by a second group of *Wnt* genes (*NvWnt5*, *-6* and *-8*) in the endoderm (Fig. 2a′–o′). *NvWnt5* is expressed in the most oral region of the

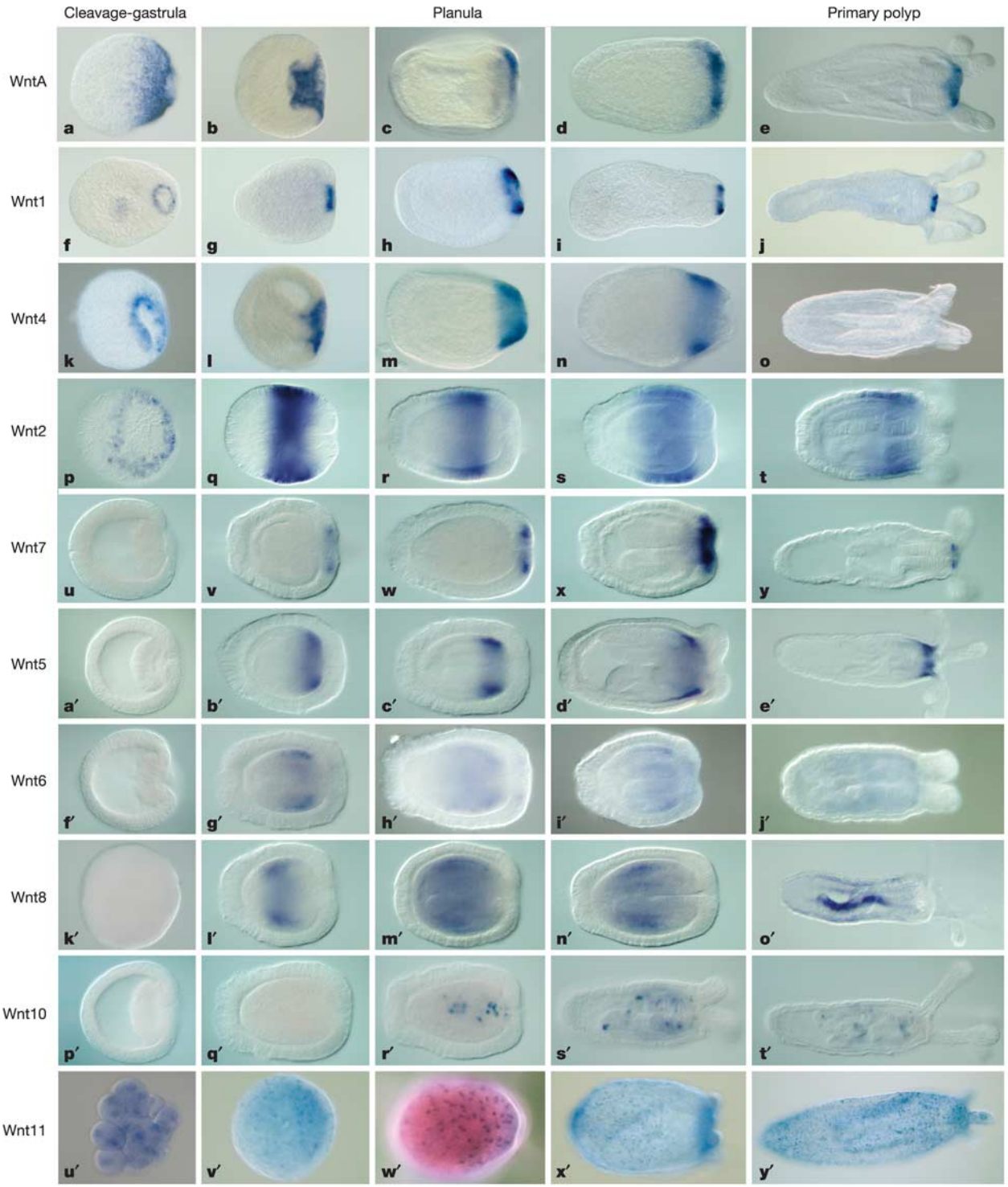

Figure 2 Expression of *N. vectensis Wnt* genes during embryogenesis. Whole-mount *in situ* hybridizations reveal ectodermal (**a**–**y**), endodermal (**a**′–**o**′) and cellular (**p**′–**y**′) expression patterns in overlapping domains. **a**–**e**, *NvWntA* exhibits bilaterality in planulae. **f**–**j**, *NvWnt1* expression at the oral pole. **k**–**o**, *NvWnt4* expression in a broad domain below the blastopore. **p**–**t**, Stripe-like expression of *NvWnt2* in the middle of the planula. **u**–**y**, *NvWnt7* expression around the oral pole from mid gastrulae on. **a**′–**e**′, *NvWnt5* expression in the oral endoderm from late gastrulae on. **f**′–**j**′, *NvWnt6* expression below the blastopore and in endodermal derivatives. **k**′–**o**′, *NvWnt8* expression in mid-body endoderm and polyp's primary mesenteries. **p**′–**t**′, *NvWnt10* expression in individual cells near oral pole and in primary mesenteries. **u**′–**y**′, *NvWnt11* expression in individual cells.

Table 1 **Distribution of *Wnt* gene subfamilies**

	WntA	*Wnt1*	*Wnt2*	*Wnt3*	*Wnt4*	*Wnt5*	*Wnt6*	*Wnt7*	*Wnt8*	*Wnt9*	*Wnt10*	*Wnt11*	orphan *Wnts**
Cnidaria	1	1	1	1	1	1	1	2	2		1	1	
Ecdysozoa													
Insects	1	1	0	0	0	1	1	1	0	1	1	0	1
Nematode	0	1†	0	0	0	1	?	?	0	?	0	0	3
Lophotrochozoa													
Turbellaria						1†							
Polychaete	1	1	1†		1			1†		1†	1†		
Mollusc	1	1†	1					1†			1†		
Deuterostomia													
Amphioxus		1	1	1	1	1	1	2	1	2	1	1	
Human	0	1	2	2	1	2	1	2	2	2	2	1	1
Ur-Eumetazoa	1	1	1	1	1	1	1	1	1	?	1	1	

*There is an additional *Wnt* gene from *D. melanogaster* in the database described as *DmWnt8* (accession number Q9VFX1) that shows, however, no orthology to any of the conserved subfamilies and lacks a large set of conserved features of Wnt ligands, including some of the conserved cysteine residues. Similarly, human *Wnt16* and three *Wnt* genes from *C. elegans* exhibit no orthology to any of the conserved subfamilies. We call these genes 'orphan *Wnts*'.
†Sequences cluster within the conserved subfamilies[2,10], but were not used in the phylogenetic analyses shown in Fig. 1 (see Methods).

endoderm (Fig. 2a′–e′, Supplementary Fig. S6), while *NvWnt6* and *-8* are expressed in more aboral domains, which differentiate into endoderm and mesenteries at the late planula and polyp stage (Fig. 2b′–j′). Although the boundaries between gene expression domains are not sharp and overlap one another, there are distinct regional differences in their expression along the oral–aboral axis reminiscent of *Hox* gene expression in bilaterian animals.

Two of the *Wnt* genes are expressed only in individual cells: *NvWnt10* in the endoderm (Fig. 2p′–t′) and *NvWnt11* in the ectoderm (Fig. 2u′–y′). This expression pattern suggests a more direct role of these genes in cell type specification. Two of the *Wnt* genes also show asymmetrical expression in an axis perpendicular to the oral–aboral axis. *NvWnt4* expression is excluded from one portion of the blastopore (Fig. 2k) and *WntA* is skewed to one side of the blastopore (Fig. 2c, Supplementary Fig. S4). Expression behaviour similar to that of *NvWnt4* was also observed for *NvFkh*[13] and might reflect unequal progression of the invagination along the blastopore margin. These data add to a growing body of evidence from *Hox* and *TGFβ* genes (*BMP4/Dpp* and *GDF5*) that anthozoan cnidarians have a secondary body axis (the 'directive' axis) with a definitive polarity that can now be seen not only at the morphological[14] but also at the molecular level[3,15].

Notably, a distinct *Wnt* expression can also be found at the side of blastopore formation and in the gastrulating endoderm in most basal deuterostomes and protostomes investigated so far[7,16,17]. *Wnt* genes are also co-expressed together with the transcription factors *Brachyury*, *Forkhead*, *Snail*, *Notch* and *Caudal* and represent a conserved cassette of genes that may define the blastoporal signalling centre[7,18]. That some of this group of transcription factors is also present in cnidarians[13,19,20] indicates that this blastoporal patterning system is not a deuterostome or protostome innovation but an inheritance from the basal diploblastic animals.

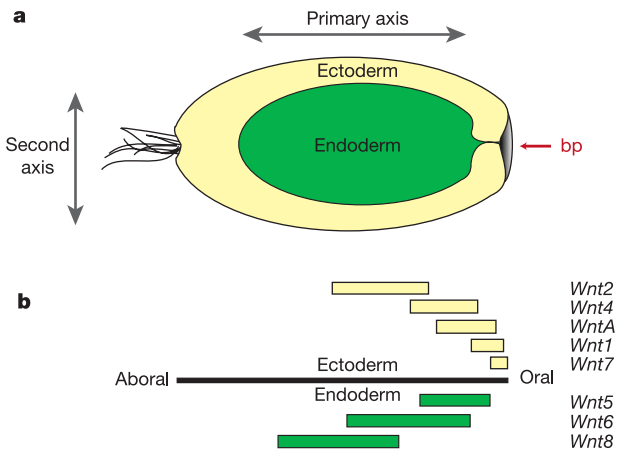

Figure 3 Overlapping expression domains of *Wnt* genes in a *N. vectensis* planula. **a**, The blastopore (bp) marks the oral end, a ciliary tuft the aboral end of the planula. Tentacles form after metamorphosis at the oral end. **b**, Ectodermal (yellow) and endodermal (green) *Wnt* genes are expressed in staggered arrays along the oral–aboral axis.

We propose that *Wnt* genes of this ancient blastoporal signalling centre gave rise to various mesodermal and neuro-ectodermal derivatives in the lophotrochozoan, ecdysozoan and deuterostome lineages. In support of this, in deuterostomes the group of *Wnt* genes expressed in the endoderm of *N. vectensis* (*NvWnt5*, *-6*, and *-8*) can be found expressed in the developing mesoderm with overlapping expression domains along a ventral-posterior direction[21–23]. These similarities indicate a close link between endoderm and mesoderm during gastrulation and a function of this ancient cluster of *N. vectensis Wnt* genes in mesoderm evolution. By comparison, the group of *Wnt* genes expressed in the ectoderm of *N. vectensis* (*NvWnt1*, *-2*, *-4* and *-7*) has a strong bias towards neuro-ectodermal expression domains. Particularly in the developing vertebrate nervous system there are striking similarities along a dorsal-anterior axis[24–27], which lead us to the hypothesis that the nervous system was patterned by an ancient set of ectodermal *Wnt* genes, probably in a staggered array along an ancient oral–aboral axis.

Thus our data indicate that in the gastrulation of the sea anemone *N. vectensis*, a blastoporal signalling centre is active, where nearly all *Wnt* gene subfamilies are co-expressed. Their staggered expression along the oral-aboral axis of the planula larva (Fig. 3) suggests that *Wnt* genes probably had an ancient and primary function in gastrulation and axial differentiation, although this has been questioned recently[9]. The pattern is surprisingly reminiscent to the anterior–posterior expression of *Hox* genes in bilaterians.

Our result also points to an unexpected paradox of genome evolution: the gene diversity in the genomes of simple metazoans is much higher than previously predicted[3] and some derived lineages (flies and nematodes) have an even lower diversity of gene family members. Thus there is no simple relationship between genetic and morphological complexity. We presume that for the successful transition from single cell to multi-cellular animals a whole concert of interacting signalling molecules was required. This led to the formation of a stable signalling centre, which induced the complex cellular machinery of gastrulation, causing the ingression of cells and/or invagination of an outer body layer. This 'robust' patterning system was probably the starting point in the rapid generation of more complex animal body plans. An expansion of transcription factor families—as for instance in the case of *Hox* genes[28] in chordate and of *MADS box* genes[29] in flower evolution—was probably correlated with the later rise in morphological complexity during the Cambrian evolutionary explosion and more recent evolution. □

Methods

Isolation of *Wnt* genes from *N. vectensis*

Nested PCR was used to amplify 122–144-bp fragments of *Nematostella Wnt* genes. We used degenerate primers aimed to amplify any *Wnt* types. PCR was done on complementary DNAs reverse-transcribed from messenger RNA isolated from 12–120 h *N. vectensis* embryos. Primer combinations were as follows: 5′-TGG(GC)A(AGCT)T GGGG(AGCT)GG(AGCT)TG-3′ as forward primer in both rounds of nested PCR, and 5′-T(CT)(AGCT)CC(AG)TG(AG)CA(CT)TT(AG)CA-3′ as outer and 5′-CC(AGCT)GC (AGCT)(CT)(GCT)(AG)TT(AG)TT(AG)TG-3′ as inner reverse primer. Eleven different *Wnt* genes were thus isolated from *N. vectensis*; *NvWnt8a* was obtained from an EST project (U.T. and T.W.H.). The 3′ and 5′ ends of the corresponding genes were amplified from cDNA libraries by using vector-specific primers and gene-specific (non-degenerate) primers. Primer sequences and experimental conditions are available upon request. PCR products were cloned into the pGEM-T vector (Promega) TOPO-TA or into the pCR2.1-TOPO vector using the TOPO TA Cloning reagents (Invitrogen); all clones were sequenced on an ABI automated sequencer.

Retrieval and alignment of *Wnt* gene sequences

Wnt protein sequences were obtained through the retrieval of Wnt protein sequences listed on R. Nusse's Wnt home page (http://www.stanford.edu/~rnusse/wntwindow.html) or by database searches on NCBI, SWISSPROT as well as Sanger, and by BLASTP search at the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/). All sequences and their accession numbers are available as Supplementary Information (Table 1, Fig. 1). ClustalW was used for the protein alignments (http://www.ebi.ac.uk/clustalw/). Where available, only full-length sequences were used. *PvWntA* and *PdWntA* sequences were included because no other full-length sequences are available; the *NvWnt6* and *NvWnt8a* sequences are not yet completely full-length, but give sufficient sequence information for a reliable phylogenetic analysis. Alignments were subsequently manually improved by using alignments of Wnt domains available in PFAM (http://www.sanger.ac.uk/Software/Pfam/) or SMART (http://smart.embl-heidelberg.de/). The Wnt domain itself contains several regions of high conservation separated by less-conserved stretches of amino acids that are not particularly well aligned. Given that sequence alignment influences phylogenetic reconstruction, we explored alternative alignments of these less-conserved regions by changing the gap penalty of ClustalW. These different alignments gave essentially similar results in the phylogenetic analyses, as well as discarding positions with more than 50% gaps.

Phylogenetic analyses

Bayesian analysis was performed with MrBayes 3.0B4 (http://morphbank.ebc.uu.se/mrbayes/) using the Jones–Taylor–Thornton (JTT) model of protein evolution with invariant sites and four Gamma-distributed rates. Six chains were run for 20,000,000 generations; after a burn-in of 1,000,000 generation every 100th tree was sampled for a 50% majority consensus. In addition, ML analyses were done with TREE-PUZZLE 5.2 (http://www.tree-puzzle.de/), as well as IQPNNI 2.2 (ref. 30) (http://www.bi.uni-duesseldorf.de/software/iqpnni/). Bootstrap support values were constructed using PAUP* 4.0b (http://paup.csit.fsu.edu/), applying the MP criterion. For details see Supplementary Methods.

In situ hybridization

The procedure of the *in situ* hybridization was performed as described[13] with the following changes: Specimens were fixed in 4% MEMPFA containing 0.0625% glutaraldehyde for 3 h, and then stored in methanol at −20 °C. Hybridization of the DIG-labelled RNA probe was carried out at 44–65 °C for at least 36 h, post-hybridization washes were done in 50% formamide/2 × SSC/0.02% TritonX-100 over 8 h by raising the temperature gradually from 47 °C to 56 °C. Visualization of the labelled probe was performed using NBT BCIP (Boehringer) as substrate for the alkaline phosphatase conjugated anti-DIG antibody used in the procedure.

Received 23 August; accepted 1 November 2004; doi:10.1038/nature03158.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements This work was supported by grants from NASA and the NSF to M.Q.M. and the German Science Foundation (DFG) to U.T., B.H. and T.W.H. Some computations were carried out on the JUMP supercomputer at the ZAM/NIC of the Research Center Jülich. We thank A. Busch and C. Niehrs for critically reading the manuscript.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.Q.M. (mqmartin@hawaii.edu) and T.W.H. (holstein@uni-hd.de). The newly determined sequences are deposited at NCBI GenBank under accession numbers AY534532, AY530300–1, AY725201–4, AY687348–9, AY792510 and AY657172.

Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei

Mohamed Hijri & Ian R. Sanders

Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland

Arbuscular mycorrhizal fungi (AMF) are ancient asexually reproducing organisms that form symbioses with the majority of plant species, improving plant nutrition and promoting plant diversity[1,2]. Little is known about the evolution or organization of the genomes of any eukaryotic symbiont or ancient asexual organism. Direct evidence shows that one AMF species is heterokaryotic; that is, containing populations of genetically different