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REVIEW

# The Wnt code: cnidarians signal the way

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**Cnidarians are the simplest metazoans with a nervous system. They are well known for their regeneration capacity, which is based on the restoration of a signalling centre (organizer). Recent work has identified the canonical Wnt pathway in the freshwater polyp *Hydra*, where it acts in organizer formation and regeneration. Wnt signalling is also essential for cnidarian embryogenesis. In the sea anemone *Nematostella vectensis* 11 of the 12 known *wnt* gene subfamilies were identified. Different *wnt* genes exhibit serial and overlapping expression domains along the oral–aboral axis of the embryo (the '*wnt* code'). This is reminiscent of the *hox* code (cluster) in bilaterian embryogenesis that is, however, absent in cnidarians. It is proposed that the common ancestor of cnidarians and bilaterians invented a set of *wnt* genes that patterned the ancient main body axis. Major antagonists of Wnt ligands (e.g. Dkk 1/2/4) that were previously known only from chordates, are also present in cnidarians and exhibit a similar conserved function. The unexpectedly high level of genetic complexity of *wnt* genes evolved in early multi-cellular animals about 650 Myr ago and suggests a radical expansion of the genetic repertoire, concurrent with the evolution of multi-cellularity and the diversification of eumetazoan body plans.**
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## Cnidarians are genetically complex

The Cnidaria is an ancient metazoan phylum of diploblastic animals including freshwater polyps and hydroids, sea anemones and corals, and jellyfish. All cnidarians share the same simple body plan that is reminiscent of an early bilaterian gastrula. However, they are lacking the mesoderm and possess only two germ layers, an outer ectoderm and inner endoderm that are separated by an acellular mesogloea. Cnidaria are a sister-group to the Bilateria (Figure 1), and the fossil record reveals that cnidarians are > 500 Myr old (Chen *et al*., 2000, 2002; Conway Morris, 2000). They are of crucial importance for unravelling the origin and evolution of major signalling pathways in animal evolution.

There are two major genetic model systems for cnidarians: the well-known freshwater polyp *Hydra* (Steele, 2006) and the starlet sea anemone *Nematostella vectensis* (Holland, 2004; Darling *et al*., 2005), which was introduced by the pioneering work of Cadet Hand (Hand and Uhlinger, 1992). Recent EST projects in these and some other cnidarian taxa have revealed an astonishing and unexpected genetic complexity of cnidarians. Analyses of ESTs from the anthozoans *Acropora millepora* and *Nematostella vectensis* have lead to the identification of 16 571 non-redundant ESTs and 12 547 predicted peptides across the two species (7484 from *Nematostella* and 5063 from *Acropora* (Miller *et al*., 2005; Technau *et al*., 2005). Both data sets are far from saturation and one can estimate that anthozoan genomes are likely to contain 25 000 genes, which is in the same range as vertebrates. These would be more genes than in *Drosophila* (~14 000 protein coding genes; http://www.flybase.net/annot/release.html) or in *Caenorhabditis* (~19 000 protein coding genes, http://www.sanger.ac.uk/Projects/C_elegans/WORMBASE/current/release_notes.txt). These data are confirmed by EST data from various *Hydra* labs, the recently released genome project in *Nematostella* (DOE Joint Genome Institute; http://www.jgi.doe.gov; Daniel Rokhsar, JGI Eukaryote Program Lead) and will soon receive further input from the *Hydra* genome project. While it is unclear at present, to what extent the genomes of *Hydra* and *Nematostella* differ among each other and bilaterians, the analysis of the cnidarian gene catalogue already revealed three major findings: (i) A large number of orthologous developmental genes known to be important for bilaterian development, including the genes for Wnt, TGF-$\beta$, and FGF signalling as well as Hox, Fox, Brachyury transcription factors are present in cnidarians (Technau and Bode, 1999; Spring *et al*., 2000, 2002; Scholz and Technau, 2003; Technau and Scholz, 2003; Finnerty *et al*., 2004; Fritzenwanker *et al*., 2004; Hayward *et al*., 2004; Martindale *et al*., 2004; Torras *et al*., 2004; Extavour *et al*., 2005; Magie *et al*., 2005; Technau *et al*., 2005; Torras and Gonzalez-Crespo, 2005; Matus *et al*., 2006). (ii) Among these conserved factors, even 'mesodermal' proteins that are specific for mesoderm induction and differentiation in triploblastic bilaterian animals are present. This is striking since

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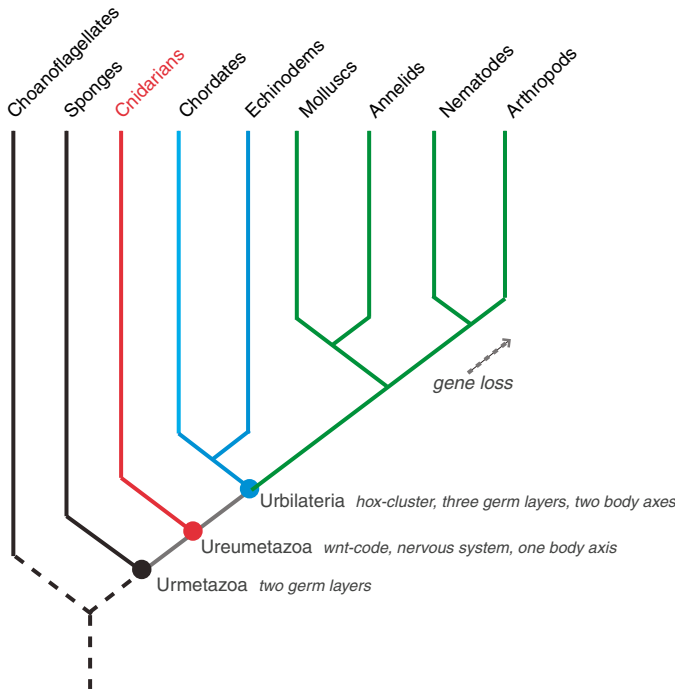


**Figure 1** Phylogenetic relationship between major metazoan clades. Molecular phylogeny supports a single origin for the animal kingdom in an organism called the urmetazoan. Sponge-like organisms probably arose from colonial single cell organisms, resembling the present-day choanoflagellates, by developing cell-to-cell signalling systems. Also the Bilateria had a single origin in a common ancestor of all 'higher' animals called the urbilaterian and splitted into echinoderms, chordates and other deuterostomes (blue) and in protostomes clades (green). Cnidarians (red) branched off the metazoan stem before the origin of the Bilateria, and represent animals with a tissue grade of organization and a nervous system (Eumetazoa).

cnidarians are diploblastic organism lacking a mesoderm. (iii) The complexity of genes in the anthozoan and hydrozoan gene set encoding developmentally regulated signalling pathways do not differ substantially from those of vertebrates. This is noteworthy, since several gene families are completely absent from the genomes of urochordates (*Ciona* and *Oikopleura*) as well as from the model invertebrates *Caenorhabditis* and *Drosophila*. Thus, a great deal of the genetic repertoire responsible for the formation of specific bilaterian body plans already existed in the common ancestor of cnidarians and bilaterians.

The molecular and genomic tools have been developed to study *Hydra* and *Nematostella* on a functional level. Besides *in situ* hybridization (ISH) protocols and the full set of the genomic tools (BAC, EST and cDNA libraries) RNAi, morpholinos, transient and stable transfection are available or under optimization procedure. The introduction of transgenic approaches to the field of cnidarian biology was a major breakthrough. Wittlieb *et al*. (2006) succeeded in producing the first stable transgenic *Hydra* by using a GFP-coding gene driven by a *Hydra actin* gene promoter that can be injected into blastomeres of two- to eight-cell *Hydra* embryos. The rate of stably integrated transgenes is about 12%, which is a yield comparable to that seen with mouse embryos.

Thus, due to their morphological simplicity and especially of their remarkable regeneration capacity cnidarians can serve as an important model to understand basic functions of Wnt signalling in gastrulation, axis formation, germ-layer specification, regeneration and cell differentiation.

## Members of cnidarian *Wnt* signalling

Initial work identified the canonical Wnt/β-catenin pathway in *Hydra* with a *wnt3a*, *dishevelled*, *gsk-3β*, *β-catenin* and *tcf/lef* (Hobmayer *et al*., 1996; Hobmayer *et al*., 2000) as well as a putative Wnt receptor *frz7* (Minobe *et al*., 2000). Recent work revealed that almost all bilaterian *wnt* gene subfamilies are present in cnidarians (Kusserow *et al*., 2005; Lee *et al*., 2006 unpublished work from the HR Bode, B Hobmayer and TW Holstein labs) suggesting that the main genes of the Wnt pathways are present in cnidarians.

### *Wnt ligands*

The genes encoding for Wnt ligands comprise a large, multi-gene family in bilaterians. In human and mouse genomes 19 *wnt* genes are present that cluster into 13 *wnt* gene subfamilies. An analysis of *wnt* sequences from various lophotrochozoan representatives revealed an additional wnt gene (*wntA*) that is not present in vertebrates (Prud'homme *et al*., 2002). From *Nematostella* a total of 14 different *wnt* genes was isolated (Kusserow *et al*., 2005; Lee *et al*., 2006, M Ritthaler, A Kusserow and TW Holstein, unpublished), representing 12 of the 13 *wnt* subfamilies (Table 1). The *Nematostella* genome only lacks the *wnt9* subfamily. There are also

**Table 1** Distribution of *wnt* gene subfamilies in the animal kingdom

| | wntA | wnt1 | wnt2 | wnt3 | wnt4 | wnt5 | wnt6 | wnt7 | wnt8 | wnt9 | wnt10 | wnt11 | wnt16 | wnt *genes orphan* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cnidarians | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | | 1 | 1 | 1 | |
| Insects | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| Nematode | 0 | 1 | 0 | 0 | 0 | 1 | ? | ? | 0 | ? | 0 | 0 | 0 | 3 |
| Annelids | 1 | 1 | 1 | | 1 | | | 1 | | 1 | 1 | | | |
| Molluscs | 1 | 1 | 1 | | | | | 1 | | | 1 | | | |
| Chordates | | | | | | | | | | | | | | |
| 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 | 1 | |

Modified from Kusserow *et al*. (2005). *wnt16* is included as a separate wnt gene subfamily. *wnt* genes from *C. elegans* and *Drosophila* that exhibit no orthology to any of the conserved subfamilies are called *orphan wnts*.

two representatives each from the *wnt7* and *wnt8* subfamilies. While *wnt8a* and *wnt8b* represent paralogous genes that probably arose from cnidarian-specific duplication events (Kusserow *et al*., 2005), the structural identity in the C terminal region of *nvwnt7A* and *nvwnt7B* suggests that these transcripts actually represent alternate splice variants from a single locus. Recent work from *Hydra* indicates that most of the *wnt* gene subfamilies found in *Nematostella* are also present in *Hydra* (unpublished work from the HR Bode, B Hobmayer and TW Holstein labs).

*An ancient wnt cluster?*

The phylogenetic relationships among all Wnt subfamilies are unclear so far. Nonetheless, a clustering of the Wnt1/6/10/9/3 subfamilies comes out in the phylogenetic analyses, which is supported by the syntenic *wnt* organization between *Drosophila* and vertebrates (Kusserow *et al*., 2005). In the *Drosophila 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*/*wnt9a* and *wnt3*/*wnt9b* are closely linked suggesting that *wnt* genes 1/6/10/9/3 represent an ancestral cluster of *wnts* that originated in the evolution of the common ancestor of cnidarians and bilaterians. This view receives further support by a first finding that an analysis of the trace files from the *Nematostella* genome reveals close genomic linkage between *nvwnt6* and *nvwnt10* (Y Nakamura and TW Holstein, unpublished). Establishing to what extent the *wnt* genes were clustered in *Nematostella* will need to await complete assembly and evaluation of the sea anemone genome.

*The origin and loss of wnt genes*

The fact that nearly all of the w*nt* genes found in vertebrates could be also identified in *Nematostella* (Kusserow *et al*., 2005) was surprising. It demonstrates that the diversification of *wnt* genes already occurred in the ureumetazoans, that is before cnidarians and bilaterians diverged. No *wnt* genes have been described so far from unicellular eukaryotes, neither from cellular slime molds (*Dictyostelium discoideum*) nor from choanoflagellates (King *et al*., 2003), unicellular and colonial Protozoa that are closely related to Metazoa. While as yet no data are available from sponges, which probably diverged before the origin of the eumetazoan ancestor, we presume that the appearance and diversification of *wnt* genes itself was linked to the origin and evolution of multi-cellular animals from single-cell (protozoan) ancestors.

A comparison of the cnidarian *wnt* genes with genomes data from other bilaterians also reveals that a major gene loss occurred in the protostome lineage of bilaterian evolution. In insects and nematodes only seven and five *wnt* genes can be identified in the *Drosophila* and *Caenorhabditis* genome. This clearly shows that there is no simple correlation between the increase in morphological complexity and the repertoire in genes of genomes (Kusserow *et al*., 2005).

*Receptors and intracellular components of Wnt signalling*

In addition to the Wnt ligands and extracellular modulators (see below), other components of both canonical and non-canonical Wnt signalling cascades have been identified in *Nematostella* and *Hydra*. These include orthologs of $\beta$-catenin (Hobmayer *et al*., 1996), GSK-3$\beta$, Tcf/Lef, Dsh, APC and Axin from the Wnt/$\beta$-catenin pathway (Technau *et al*., 2005), Flamingo, Van Gogh, and JNK from the Wnt/PCP (planar cell polarity) pathway; and *Cam*KII and PKC from the Wnt/$Ca^{2+}$ pathway (Lee *et al*., 2006), but also for casein kinase 1$\alpha$, 1$\delta$, 1$\gamma_2$, 1$\gamma_3$ and 1$\varepsilon$ (T Lengfeld and TW Holstein, unpublished), which are all known to act in Wnt signalling (Price, 2006; Strutt *et al*., 2006). Furthermore, the evidences indicating *Nematostella* and *Hydra* have several kinds of receptors for canonical and non-canonical Wnts have now started to accumulate. Although it remains to be clarified whether cnidarians have a LRP5/6-related gene (Guder *et al*., 2006), at least six Frizzled (Fzd) receptor genes in *Nematostella* and one *fzd* gene, which seems most closely related to human *fzd7*, in *Hydra*, have been identified (Minobe *et al*., 2000). *In silico* analyses reveal that *Hydra* seems to have two more *fzd* genes resembling *fzd4*/*9*/*10* and *fzd5*/*8* subfamily members (H Watanabe and TW Holstein, unpublished). In addition, *Nematostella* and *Hydra* have also related genes for other Wnt

receptors, for example the receptor tyrosine kinase gene *ror*, which is known as a non-canonical Wnt receptor and to be implicated in non-canonical Wnt signal-mediated inhibition of canonical pathway. *In silico* analysis also reveals a *Nematostella* and *Hydra wntless* gene (H Watanabe and TW Holstein, unpublished) encoding for a transmembrane protein that is essential for Wnt secretion (Banziger *et al*., 2006; Bartscherer *et al*., 2006). In summary, one can conclude that the existence of multiple Wnts and their receptors in cnidarians indicates an expansion of these gene families in early metazoan evolution and before the formation of bilaterian body plans (Kusserow *et al*., 2005; Miller *et al*., 2005; Technau *et al*., 2005).

## Wnt signalling during early embryogenesis

Most data on Wnt signalling during cnidarian embryogenesis where performed in *Nematostella* and reveal a striking overlapping localization of nearly all *wnt* genes at the site of the blastopore during and after gastrulation of the embryo. They indicate an ancient function for Wnt signalling in gastrulation and axial patterning.

*An ancient function of Wnt signalling in axial patterning*
The expression domains for the *Nematostella wnt* genes have been determined by ISH and they exhibit a characteristic pattern (Figure 2) during embryogenesis (Kusserow *et al*., 2005). Most are expressed along the oral–aboral axis, and they are associated with the blastopore during gastrulation and/or to the oral region of the planula or polyps (Kusserow *et al*., 2005). Each subfamily of *wnt* genes is also restricted to one of the two body layers, the ectoderm or the endoderm (Figure 2). Five *wnt* genes (*nvwntA*, *1-2*, *4* and *7*) are expressed in staggered domains in the ectoderm (Figure 2a and b), and they span the entire oral–aboral axis except for the aboral pole itself. *nvwntA* is expressed at the oral end and its expression commences in the early gastrula as a broad expression domain defining the site of gastrulation. *nvwnt2* is expressed at the most aboral end as a large stripe in the middle of the embryo, while *nvwnt1*, *4* and *7* expressions are in between. A similar distribution of gene expression is seen by a second group of *wnt* genes (*nvwnt5*, *6* and *8*) in the endoderm with *nvwnt5* at the most oral end, and *nvwnt8* at the most aboral end. The boundaries between gene expression domains are not sharp but overlap with each other. These distinct regional and germ-layer specific expression patterns of the *wnts* suggest that the ancestral role of these genes was in specifying position along the main body axis (Kusserow *et al*., 2005).

*A wnt code, but not a hox code patterns the ancient body axis*
The distinct regional expression of *wnts* along the oral–aboral axis is highly similar to the *hox* gene expression pattern along the anterior–posterior axis in bilaterian animals and can be defined as the 'Wnt-code',

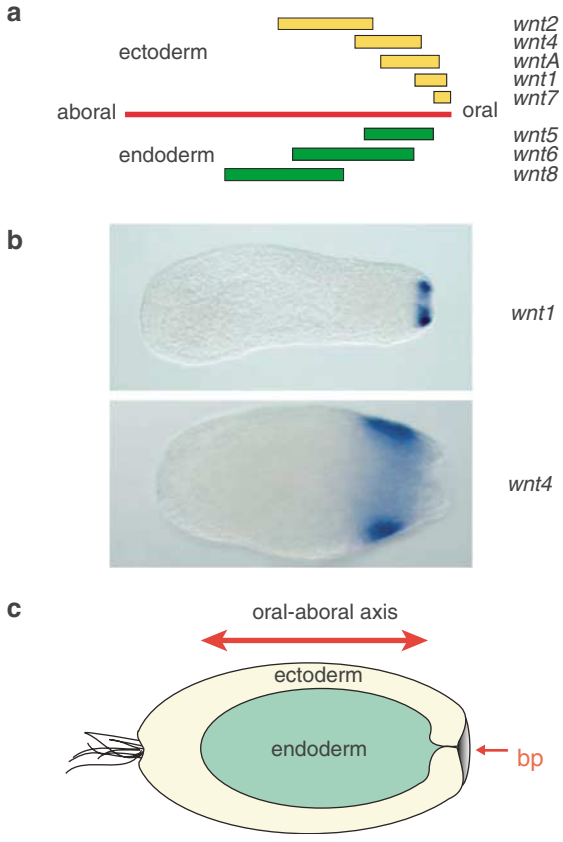


**Figure 2** The Wnt code. Overlapping expression domains of *wnt* genes in a *Nematostella vectensis* planula reveal expression domains in staggered arrays along the oral–aboral axis that are schematically shown in (**a**) and visualized by ISH for the *wnt1* and *wnt4* gene representatively, in (**b**). (**c**) Diploplastic organization of a planula larva, with the blastopore (bp) marking the oral end and a ciliary tuft the aboral end. Tentacles form after metamorphosis at the oral end, which is homologous to the hypostomal and head organizer region in the freshwater polyp *Hydra*.

accordingly. There is more evidence that the Wnt-code was evolved in early metazoan animals before axial patterning via the *hox*/*parahox* cluster was fixed, though *hox* genes first were postulated to fulfil similar axis-determining functions in cnidarians (Finnerty *et al*., 2004). However, new data reveal that no complete *hox* code exists in cnidarians. Although some if not all cnidarians possess a limited number of bilaterian-like anterior *hox* genes, many of these genes are paralogs and they do not represent equivalents of the *hox* cluster (Chourrout *et al*., 2006; Kamm *et al*., 2006). One hypothesis is that *hox*/*parahox* clusters originated from the duplication of an ancient *protohox* cluster with anterior and posterior *hox*/*parahox* genes (Brooke *et al*., 1998; Ferrier and Holland, 2001; Brooke and Holland, 2003; Garcia-Fernandez, 2005a, b). However, other findings propose the *protohox* cluster may have only consisted of two anterior genes, while the non-anterior genes most probably appeared independently in the *hox* and *parahox* clusters after the separation of bilaterians and cnidarians (Chourrout *et al*., 2006; Kamm *et al*., 2006).

The fact that not the canonical *hox* system, but *wnt* genes appear to be mandatory for axial patterning in *Nematostella* arises a number of interesting questions. Are the axial expression domains of *wnt* genes reflected by the syntenic organization of *wnt* genes, similar to the bilaterian *hox* cluster? Is there a regulative hierarchy of *Nematostella wnt* gene expression during *Nematostella* embryogenesis? Is there any fixed order of *Nematostella wnt* genes that signal through the canonical, the Wnt/PCP, and Wnt/$Ca^{2+}$ pathways? And how are the various Wnts related to the receptors and co-receptors of Wnt signalling?

*Wnt signalling in gastrulation*

The blastoporal signalling centre in cnidarian embryos is on the molecular level reminiscent of the *Hydra* organizer (see below) and it is was therefore proposed that the synexpression group defined by the *wnts*, *brachyury*, *forkhead* and *chordin* represents an ancestral system responsible for axial patterning and regulation of cell movements and differentiation at the site of the blastopore (Holland, 2002; Holland *et al*., 2001; Kusserow *et al*., 2005). Studies on the functional level in *Nematostella* support this view and show that canonical Wnt signalling is essential for the gastrulation process.

$\beta$-Catenin becomes differentially stabilized along the oral–aboral axis and translocated into nuclei in cells at the site of gastrulation, the blastopore (Wikramanayake *et al*., 2003). This was shown by an antibody against $\beta$-Catenin and was further confirmed using an endogenous and a *Xenopus* $\beta$-catenin-GFP fusion protein. $\beta$-Catenin was degraded in one half of the blastula and translocates into the nuclei of the other half, the future blastoporal side. When an activated form of *Xenopus* GFP tagged $\beta$-catenin (i.e., a form where the GSK-3$\beta$ CK-1 phosphorylation sites had been mutated) was injected, nuclear translocation of $\beta$Cat-GFP was seen in all cells of the blastula. When lithium chloride was applied (which blocks GSK-3$\beta$-mediated degradation of $\beta$-catenin), embryos exhibit extended gastrulation phenotypes, that is they form elongated planula larvae and fail to form tentacles. This demonstrates the importance of $\beta$-catenin in cnidarian gastrulation and is probably related to the well-known expansion of dorsal mesoderm in *Xenopus* embryos after LiCl treatment.

At this point again a number of questions arise: How is Wnt/$\beta$-catenin signalling in *Nematostella* embryos induced? An analysis of the function of *gsk-3β* during gametogenesis indicates that an inhibition of Wnt signalling is crucial for oogenesis (Rentzsch *et al*., 2005). Transcripts of *fzd*, *β-cat*, and *tcf* are maternally supplied and persist throughout embryogenesis (Frobius *et al*., 2003), while *wnt* transcripts appear at the onset of gastrulation in *Nematostella* and other cnidarians (Kusserow *et al*., 2005; Lee *et al*., 2006; Plickert *et al*., 2006). However, it remains unclear whether one of the *wnt* genes is involved the early steps of embryogenesis. Here, it will be particularly exciting to investigate the function of *nvwnt11* (Kusserow *et al*., 2005). In *Xenopus* it was recently shown that the initiating signal that localizes $\beta$-catenin to dorsal nuclei is not Wnt-dependent as previously assumed but depends on Wnt11 as the initiating signal (Jessen and Solnica-Krezel, 2005; Tao *et al*., 2005). It is also crucial to clarify the localization of other intracellular Wnt signalling components (Dsh, GSK-3$\beta$ Axin), as well as that of Wnt-responsive genes, for example *chordin* and *brachyury* (Scholz and Technau, 2003; Rentzsch *et al*., 2006). In that respect it should be pointed out that a *Nematostella brachyury* ortholog, *NemBra1*, is expressed at the site where gastrulation starts, together with *wnts* and *β-catenin*, suggesting a feedback loop between *wnt* and *brachyury* (Holstein *et al*., 2003). Also *chordin* mRNA first appear at the blastopore (Matus *et al*., 2006), reflecting conserved coupling of BMP and Wnt signalling at the invagination site.

## Wnt signalling during organizer formation and regeneration

Head regeneration in *Hydra* is governed by the reconstitution of the head organizer. *Hydra*'s head organizer is located at the apical tip of the hypostome and was described for the first time by Browne (1909). When this hypostomal tissue is transplanted to ectopic positions along the body column of a host polyp it organizes a secondary body axis by recruiting host tissue (Technau *et al*., 2000; Broun and Bode, 2002; Broun *et al*., 2005). The hypostome is homologous to the blastopore of the cnidarian embryo and can be compared to the vertebrate organizer based on a conserved set of genes expressed in both organizers.

*Wnt signalling in the Hydra head organizer*

The major players in the *Hydra* head organizer seem to be the *wnt* genes and there is evidence of $\beta$-catenin-dependent signalling during head induction (Hobmayer *et al*., 2000), and of $\beta$-catenin-independent signalling during tentacle and bud morphogenesis (I Philipp, F Rentzsch, TW Holstein, and B Hobmayer, unpublished). The expression patterns of the members of the canonical Wnt signalling pathway are suggestive and indicate a function for the Wnt/$\beta$-catenin pathway in the maintenance and establishment of the head organizer in adult *Hydra*. New data using recombinant HyWnt3a support this notion (H Watanabe, S Özbek, and TW Holstein, unpublished). *hywnt3a* is expressed in a small cluster of ectodermal and endodermal epithelial cells at the apical tip of the hypostome, at the site of the head organizer (Figure 3b) (Hobmayer *et al*., 2000). *hytcf* is also expressed in the hypostome, but in a broader domain than *wnt3a*, and with a graded distribution highest at the apex. *hydsh* and *hygsk-3β* are uniformly expressed throughout the polyp at low levels, although *hygsk-3β* transcripts are absent in the foot region (Hobmayer *et al*., 2000; Rentzsch *et al*., 2005). This pointed to susceptibility for Wnt signalling in areas distant from the wnt ligand source. Accordingly,

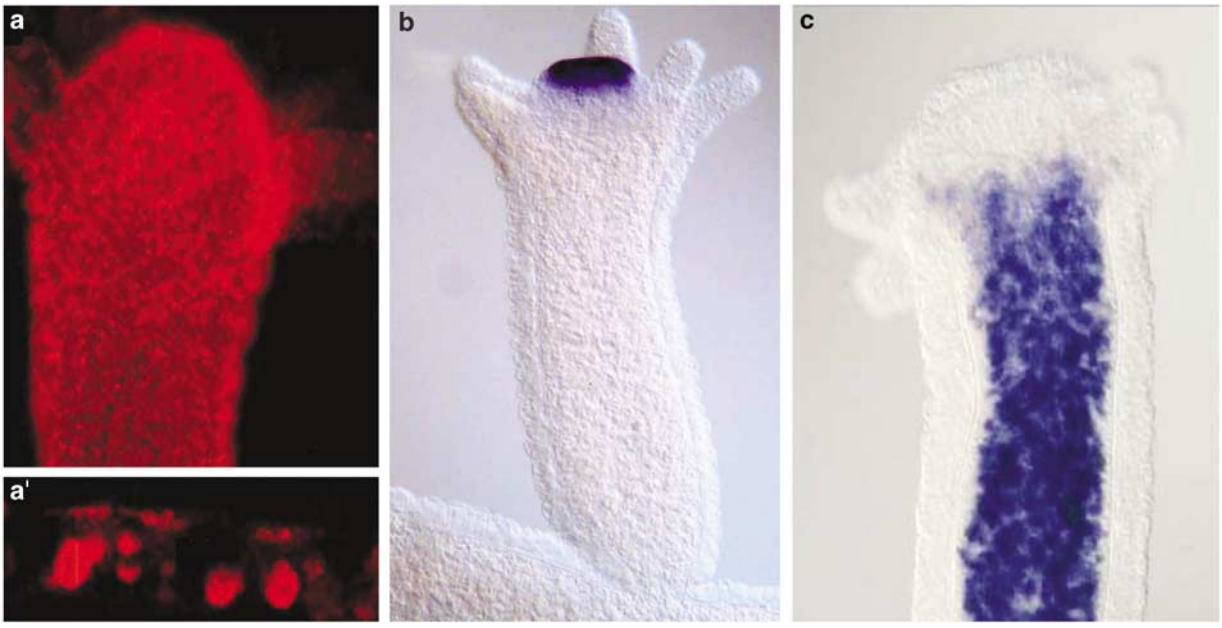


**Figure 3** The canonical Wnt pathway in Hydra. Strong nuclear localization of *β*-catenin in the hypostomal and head organizer region (**a**) and in epithelial cells (**a′**) is revealed by staining with an antibody against the C-terminal region of *Hydra β*-catenin (Cramer von Laue, 2002). ISH demonstrates an inversely related expression pattern of *wnts* (**b**) and *dkk* (**c**): *hywnt3a* transcripts are localized at the tip of the hypostome (Hobmayer *et al*., 2000), while the Wnt-anatagonist *hydkk1/2/4* is expressed in the body column (Guder *et al*., 2006).

*hyβ-catenin* transcripts are expressed uniformly throughout the polyp at low levels (Hobmayer *et al*., 2000), also consistent with an additional function for this protein in cell adhesion. Hy*β*-cat protein is localized at the apical membrane and in nuclei of epithelial cells. However, cells in the hypostome have much higher levels of nuclear *β*-catenin than cells in the body column (Figure 3a) (Cramer von Laue, 2003; Broun *et al*., 2005) indicating that Wnt signalling is active in the hypostome.

Wnt/*β*-catenin signalling is also active during bud formation. Here, *hytcf* and *hyβ-cat* expressions are upregulated prior to *hywnt3a* expression in a broad area of the body column in the region where the bud will emerge. *hytcf* expression later becomes restricted to the hypostome of the new polyp, similar to its expression domain in adult heads. *hyβ-cat* expression remains high in the apical end of the bud until the new polyp detaches (Hobmayer *et al*., 2000). The strong upregulation of *hydsh* during tissue evagination indicates an additional function in the PCP signalling pathway. In summary this set of data demonstrates an early instructive function of Wnt signalling in formation of a secondary body axis, preceding the regulated cell movements during bud evagination.

### *Activation of Wnt/*β*-catenin signalling*

The function of Wnt signalling in axial patterning of *Hydra* has been recently elegantly demonstrated by pharmacological experiments using alsterpaullone, a kinase inhibitor that specifically blocks GSK-3*β* function in *Hydra* (Broun *et al*., 2005). Earlier experiments using LiCl treatment of *Hydra* (Hassel *et al*., 1993) already indicated that an inhibition of GSK-3*β* activity (Klein *et al*., 1996) can stimulate head formation. Alsterpaullone treatment increases *β*-catenin levels in all cells of the adult polyp. In untreated polyps, the concentration of *β*-catenin in cells of the body column is maintained at low levels and nuclear *β*-catenin is largely restricted to cells in the hypostome (Figure 3a). Blocking GSK-3*β* activity with alsterpaullone increases intracellular *β*-catenin levels and the accumulation of *β*-catenin in nuclei (Broun *et al*., 2005). Alsterpaullone treatment also causes an expansion of the normal expression domains of *hytcf* and *hybra1*, the *Hydra brachyury* ortholog and induces numerous spots of *hywnt3a* expression seen along the body column (Figure 4c and d). This accounts for the findings in transplantation experiments showing that alsterpaullone-treated tissue exhibited an increased capacity to induce secondary body axes (Broun *et al*., 2005). Alsterpaullone-treated *Hydra* form numerous ectopic tentacles and finally heads along the body column (Figure 4a), this reflects the hierarchical order of Wnt-dependent processes in head (organizer) formation. It should be also emphasized that alsterpaullone has a similar effect in other cnidarians. In *Nematostella* embryos young polyps form additional tentacles at the aboral end and numerous tentacles at along the body column at higher alsterpaullone concentrations (Figure 4b). Also data from *Hydractinia echinata*, a colonial marine hydrozoan species, support the function of Wnt signalling in axial patterning. Inhibition of GSK-3*β* activity with alsterpaullone causes ectopic axes in embryos and the formation of ectopic tentacles in adult polyps of the colony (Muller *et al*., 2004a, b; Plickert *et al*., 2006; Teo *et al*., 2006). In *Hydractinia*, Wnt/*β*-catenin signalling

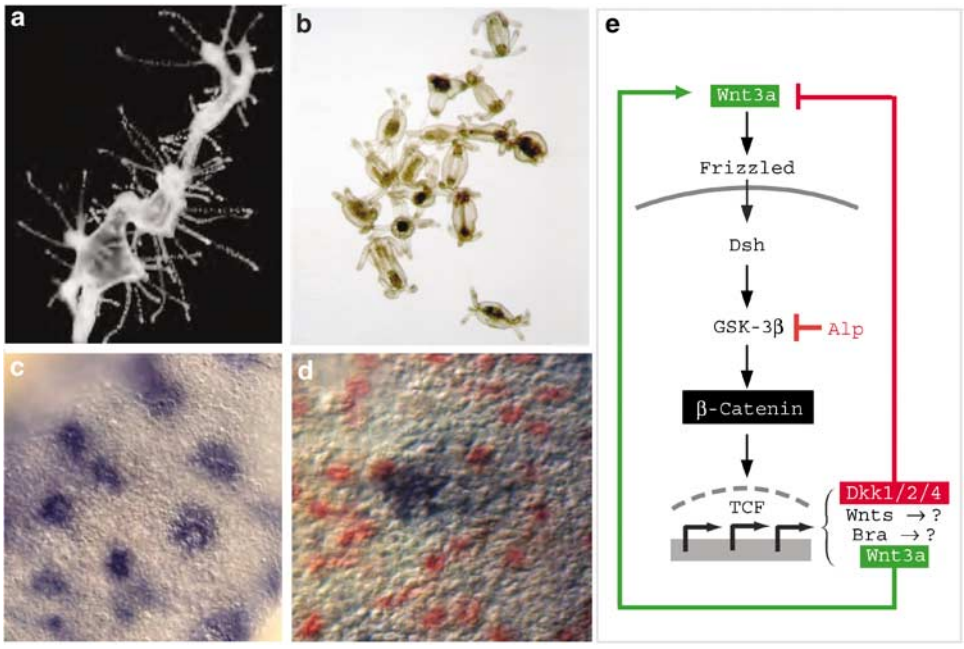


**Figure 4** Activation of the canonical wnt pathway by alsterpaullone treatment induces secondary head structures in *Hydra* (**a**) and *Nematostella* planulae (**b**). ISH analysis reveals numerous *hywnt3a* expression domains in alsterpaullone-treated animals (**c**) at sites where *hydkk1/2/4A* transcripts become successively downregulated (**d**) (Guder *et al*., 2006). (**e**) Model suggesting an autocatalytic feedback loop in the activation of the canonical wnt pathway. The activation of canonical Wnt signalling – directly or indirectly – can also induce non-canonical *wnts* and *brachyury*. Dkk1/2/is activated during regenerating probably similar as Wnt1 induced Dkk1 activation in mammals, which have multiple TCF4 sites in the dkk-1 gene promoter (Gonzalez-Sancho *et al*., 2005). The Dkk-1 gene can be transcriptionally silenced by CpG island promoter hypermethylation in human colon cancer cell lines (Aguilera *et al*., 2006). How dkk1/2/4 is downregulated is currently is currently unknown.

also promotes stem cell fate determination (Teo *et al*., 2006).

*Wnt/β-catenin signalling during regeneration*

During *Hydra* head regeneration, *tcf* and *β-catenin* are significantly upregulated prior to *wnt3a*, showing similar kinetics like in bud formation. All these genes are expressed within 1 h after decapitation in a broad domain at the apical site of the regenerating tip. *hywnt3a* becomes successively restricted to a small region at the outermost tip of the regenerate (Hobmayer *et al*., 2000). *hydsh* expression is also stimulated, but remains confined to the site of tentacle emergence and is involved in the activation of non-canonical Wnt-pathways (I Philipp, R Aufschnaiter, F Rentzsch, TW Holstein, and B Hobmayer, unpublished). In contrast, *hygsk-3β* expression remains uniform along the body column (Rentzsch *et al*., 2005). The characteristic expression of various *wnt* genes and pharmacological intervention with *β*-catenin and JNK inhibitors indicate a hierachy of Wnt signalling in head regeneration (HR Bode and TW Holstein, unpublished; I Philipp, R Aufschnaiter, F Rentzsch, TW Holstein, and B Hobmayer, unpublished; see also He and Axelrod, 2006).

A dramatic upregulation of Wnt/*β*-catenin signalling also occurs during *de novo* pattern formation in reaggregates. *Hydra* polyps can be dissociated into suspensions of single cells so that the original polarity of the animal is completely disorganized (Technau *et al*., 2000). Within 24 h ectoderm and endoderm form, after that new head organizers form *de novo*, organizing the surrounding tissue into new body axes that separate into individual polyps (Technau *et al*., 2000; Holstein *et al*., 2003). Similar to bud formation and head regenerates, *hytcf* and *hyβ-cat* are homogeneously expressed before *hywnt3a* in early reaggregates (24 h) and are later downregulated except in regions where new head organizers are forming. *hywnt3a* is first expressed in small clusters of 10–20 endodermal cells, corresponding to the minimal number of cells that can act as an organizer (Hobmayer *et al*., 2000; Technau *et al*., 2000). These spots of *hywnt3a* increase in size, and finally become localized to the tip of the hypostome of new polyps (Hobmayer *et al*., 2000; Technau *et al*., 2000), a kinetics that reflects the self-establishment and stabilization of a signalling centre.

## A modulation of Wnt signalling in cnidarians?

A number of intracellular and extracellular regulators of Wnt signalling exist in vertebrates. The cytoplasmic regulators include GSK-3*β*, Axin, and Dsh and they all act on the stability of localization of *β*-catenin (Miller, 2002). On the extracellular level canonical Wnts are sensitive to the action of other non-canonical Wnts that interfere with the canonical pathway (Tree *et al*., 2002; Veeman *et al*., 2003; Logan and Nusse, 2004; He and

Axelrod, 2006). The strongest modulating effect, however, is due to secreted antagonists of Wnts, such as the secreted Frizzled-related protein (sFRP) family, Wnt inhibitory factor (WIF), Cerberus, and the Dickkopf (Dkk) family, which inhibit Wnt signalling by either binding directly to Wnts or by binding to the Wnt receptor complex (Davidson *et al*., 2002; Kawano and Kypta, 2003; Mao and Niehrs, 2003). Notably, Wnt antagonists are absent from the *Drosophila* and *Caenorhabditis elegans* genomes and were thought to represent deuterostome-specific genes. Members of the sFRP, WIF and Dkk families of Wnt antagonists have now been identified in the *Hydra* and *Nematostella* genomes.

Eight sFRPs are known from vertebrates (Jones and Jomary, 2002), and the sFRP sequence present in *Nematostella* and *Hydra* can be a sFRP 1/2/5 ortholog. From the four vertebrate *dickkopf* gene subfamilies (*dkk-1* to *dkk-4*), two gene subfamilies with homology to the *dkk* gene family have been cloned from *Hydra* and *Nematostella*. Phylogenetic analyses group two *Nematostella* sequences within the *dkk-3* subfamily, forming a sister-group to the single *Dkk-3*-related-gene identified in *Hydra* and in the coral *Acropora millepora* (Fedders *et al*., 2004; Guder *et al*., 2006). Similar to vertebrates, the *dkk-3* subfamily seems not to be involved in the modulation of canonical Wnt signalling. In *Hydra*, *hydkk-3* is expressed in nematocytes at late stages of nematocyte differentiation, a neuronal derivative of a stem cell line also giving rise to neuronal precursors (Fedders *et al*., 2004). This is reminiscent of vertebrates, where Dkk-3 is also considered to be involved in neuronal differentiation.

The third *Nematostella dkk* sequence, *nvdkk1/2/4*, which clusters with further *Hydra dkk* sequences, *hydkk1/2/4-A* and *-C*, are proposed to be putative precursors to the *dkk1/2/4* subfamily found in vertebrates (Augustin *et al*., 2006; Guder *et al*., 2006). *hydkk1/2/4-A* and *hydkk1/2/4-C* are co-expressed in gland cells. Both transcripts are absent in the head in a pattern inverse to that of *hywnt3a* (Figure 3e). While *hydkk1/2/4-A* is expressed throughout the gastric region (Guder *et al*., 2006), *hydkk1/2/4-C* has a graded expression pattern with a high level of transcripts just below the tentacle zone (Augustin *et al*., 2006). Two sequences reported as orthologs of *Dkk-1* and *Dkk-4* were also found in an EST collection from the tentacle of the jellyfish, *Cyanea capillata* (Yang, 2003).

Blocking the activity of GSK-3$\beta$ in *Hydra* caused a drastic downregulation of *hydkk1/2/4A* expression in the gastric tissue starting from *hywnt3a* expression domains (Figure 4e) (Guder *et al*., 2006) demonstrating negative regulation by Wnt signalling. Nevertheless, overexpression studies of *hydkk1/2/4A* in *Xenopus* embryos suggest that it is a functional ortholog of vertebrate *Dkk1* and *Dkk4*, and that it is capable of inhibiting Wnt signalling (Guder *et al*., 2006).

In *Nematostella*, *nvDkk1/2/4* is first expressed at the gastrula stage in the aboral ectoderm, at the pole opposite that of the *nvwnt* expression domains (Lee *et al*., 2006), suggestive of a role in general limiting the range of Wnt activity. In late planula larvae and early polyps, *nvDkk1/2/4* is also expressed in the endoderm of the developing mesenteries but is still absent from the oral-most regions of the animal (Lee *et al*., 2006). In addition to the *sFRP* and *Dkk* sequences, an anthozoan ortholog of another vertebrate Wnt antagonist, *WIF-1*, has been identified in EST data sets from *Nematostella* and *Acropora* (Technau *et al*., 2005). To what extent these potential antagonists regulate Wnt signalling in cnidarians, will need to be established experimentally. One must also consider the fact that an important co-receptor of Dkk-Wnt antagonism, Kremen, could not be identified in cnidarians and invertebrates so far. A LRP-like co-receptor for canonical Wnt signalling in vertebrates, has not been identified biochemically, but *in silico* analysis reveals an ortholog with low homology to LRP5/6 in *Hydra* and *Nematostella* (H Watanabe and TW Holstein, unpublished). What is clear, however, is that the presence of several classes of Wnt antagonists in cnidarians suggests that the mechanisms for modulating Wnt signalling is evolutionarily ancient, and that the complex molecular interactions regulating Wnt signalling were likely already to take place in the common ancestor of cnidarians and bilaterians, the Ureumetazoa (Figure 1).

## Conclusions and outlook

Components of all three Wnt signalling pathways are present in cnidarians, indicating that this signalling pathway is an ancient metazoan patterning mechanism. The surprising large inventory of *wnt* genes in cnidarians indicates that this developmentally important gene family was already diversified in the cnidarian–bilaterian ancestor and constitute a core process (Kirschner and Gerhart, 2005) in metazoan evolution; it seems to be a fundamental property of all animal systems. The overlapping and nested sets of expression of the *wnt* genes (*Wnt code*) in both ectodermal and endodermal layers during gastrulation, early embryogenesis and regeneration indicates that the three Wnt pathways, that is the Wnt/PCP, the Wnt/$Ca^{2+}$ and the canonical Wnt pathway act concomitantly in the patterning of the oral–aboral axis and in regeneration. This opens a number of exciting questions and perspectives for future research in this simple model system: How are the different *wnt* genes transcriptionally regulated? What is the function of the limited set of co-receptors in wnt-signalling and in the stimulation of PCP mechanisms? Does the stabilization of $\beta$-catenin require Wnt-independent mechanisms, that is, is it related to Cadherin and Protocadherin function? To fully understand the signalling function of Wnts along the oral–aboral axis it will be mandatory to identify the downstream target genes that are involved in the regulation of cell differentiation, for example the activation of mesodermal genes (Fritzenwanker *et al*., 2004; Martindale *et al*., 2004; Matus *et al*., 2006). On the other hand, it is important to unravel Wnt-inhibitory mechanisms that are a prerequisite for a number of

differentiation processes, particularly for the activation of neuronal differentiation (Onai *et al*., 2004; Lie *et al*., 2005). The genomic and experimental tool-box is available now to tackle these questions.

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