The Evolution of the Wnt Pathway

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Wnt genes are important regulators of embryogenesis and cell differentiation in vertebrates and insects. New data revealed by comparative genomics have now shown that members of the Wnt signaling pathway can be found in all clades of metazoans, but not in fungi, plants, or unicellular eukaryotes. This article focuses on new data from recent genomic analyses of several basal metazoan organisms, providing evidence that the Wnt pathway was a primordial signaling pathway during evolution. The formation of a Wnt signaling center at the site of gastrulation was instrumental for the formation of a primary, anterior–posterior body axis, which can be traced throughout animal evolution.

Wnt genes are specific for metazoans, a monophyletic group of eukaryotes with a common origin in protozoans (Ruiz-Trillo et al. 2008; Schierwater et al. 2009). So far, no *Wnt* genes have been described from any unicellular eukaryotes, neither from choanoflagellates, which are presumed to represent the common ancestor of animals (King et al. 2008), nor from other protists or fungi (Sebe-Pedros et al. 2011).

Fossil records from 580-million-year (Myr)-old Ediacaran assemblages indicate that the first metazoans were diploblastic organisms similar to modern sponges and cnidarians (Xiao and Laflamme 2009). These organisms are primarily radially symmetric (Radiata) and can be distinguished from the rest of animals showing two body axes (Bilateria). Paleontological records of the Burgess Shale assemblages document that this bilaterian diversification occurred within a short period in the lower Cambrium (Conway Morris 2000). However, major questions concerning the origin of the major phyla remain unsettled, indicating that the deep nodes in metazoan evolution are difficult to resolve (Rokas et al. 2005).

Bilaterian animals are patterned along two major body axes (Niehrs 2010): the anterior–posterior (AP) axis, which is oriented parallel to the gut and, in a perpendicular orientation, the dorsal–ventral (DV) axis. Increasing evidence suggests that the primary body axis is the AP axis, whereas the secondary body axis is the DV axis (Niehrs 2010; Holstein et al. 2011). A localized Wnt/β-catenin signaling center determines the orientation of the primary body axis in both bilaterians and non-bilaterians, indicating that the conserved Wnt signaling pathway is at the base of polarized development in all metazoans (Petersen and Reddien 2009). The secondary DV axis, which is patterned by the antagonistic BMP-Chordin network, has been conserved between vertebrate and invertebrate phyla in

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patterning the DV axis (De Robertis 2010). Prebilaterian groups like sponges and cnidarians lack a DV axis. The antagonistic BMP-Chordin network seems absent in sponges, whereas in cnidarians it is initially expressed along the primary body axis (Rentzsch et al. 2008).

Bilaterians are further split into protostomes and deuterostomes based on the site of origin of their mouth (Brusca and Brusca 2002). The protostomes comprise two major phyla designated as Lophotrochozoa and Ecdysozoa, the latter including two prominent model organisms, *Drosophila melanogaster* and *Caenorhabditis elegans*. Deuterostomes include chordates (i.e., vertebrates, urochordates, and the cephalochordate *Amphioxus*), hemichordates, and echinoderms. The transition from radial to bilateral symmetry and the origin of the two major bilaterian groups are a matter of ongoing debate with controversial hypotheses (De Robertis and Sasai 1996; Arendt et al. 2001; Martindale and Hejnol 2009; Schierwater et al. 2009). Based primarily on the expression of *Wnt*s and anterior marker genes, even the existence of a common ur-bilaterian ancestor has been questioned, positing that protostome and deuterostome lineages evolved independently from a radially symmetric cnidarian-, gastrula-like organism (Meinhardt 2002, 2004, 2006). The evolution of *Wnt* genes has therefore not only an impact for our understanding of stem cell self-renewal, cell differentiation, and apoptosis, but also for the evolution and diversification of the metazoan body plans.

ROOTS OF WNT SIGNALING IN PRE-METAZOANS

The Wnt signaling pathway is an invention of the first multicellular animals (Metazoa), because in no single-cell organism (Protozoa) has a complete Wnt signaling pathway been discovered. Nevertheless, it is possible to identify modules of this signaling pathway in several protozoans. This suggests that a successful assembly of these modules was a driving force for the formation of tissues and a signaling center at the transition of single-cell to multicellular organisms. Figure 1 summarizes the evolutionary origin of genes involved in the Wnt signaling pathway(s).

Wnt Ligands

No genes encoding for Wnt ligands have been found in any protozoan. This is different from other metazoan-specific signaling pathways, for example, Hedgehog signaling, where proteins containing a Hedge and/or Hog domain of the Hedgehog ligand can be traced back to a variety of single-cell organisms, including red algae and dinoflagellates (Ingham et al. 2011). No members of the Wnt secretion machinery like Wntless (Banziger et al. 2006; Bartscherer et al. 2006) and Porcupine (Kadowaki et al. 1996) have been found outside of the Metazoa. The invention of Wnt ligands must therefore be tightly coupled with the secretory control of Wnt proteins.

Wnt Receptors and Mediators

Wnt ligands bind to the cysteine-rich domain (CRD) of Frizzled (Fzd) receptors (Dann et al. 2001) in a ternary complex with low-density lipoprotein receptor–related proteins (LRP5/6) (Tamai et al. 2000), recruiting Dishevelled (Dsh) to the membrane. GSK3 and CK1γ phosphorylate the cytoplasmic domain of LRP5/6 (Heuberger and Birchmeier 2010), which, in turn, recruits Axin to the membrane and stabilizes β-catenin.

Wnt-related receptors have been found in the cellular slime mold *Dictyostelium discoideum*. In these social amoebae, several *Fzd*-related genes have been described, but no LRP5/6 orthologs (Eichinger et al. 2005; Prabhu and Eichinger 2006). Sixteen *Fzd*-related genes encode for a putative cysteine-rich domain (CRD) and have been denoted *Frizzled/Smoothened-like* (*fsl*) (Harwood 2008). Two members (*fslJ* and *fslK*) contain an additional KTXXXW motif, which is required for activation of the canonical pathway (Harwood 2008).

Three main components of the Wnt signal transduction cascade, that is, GSK3, CK1, and an ortholog related to β-catenin called Aardvark, have also been found in *Dictyostelium*

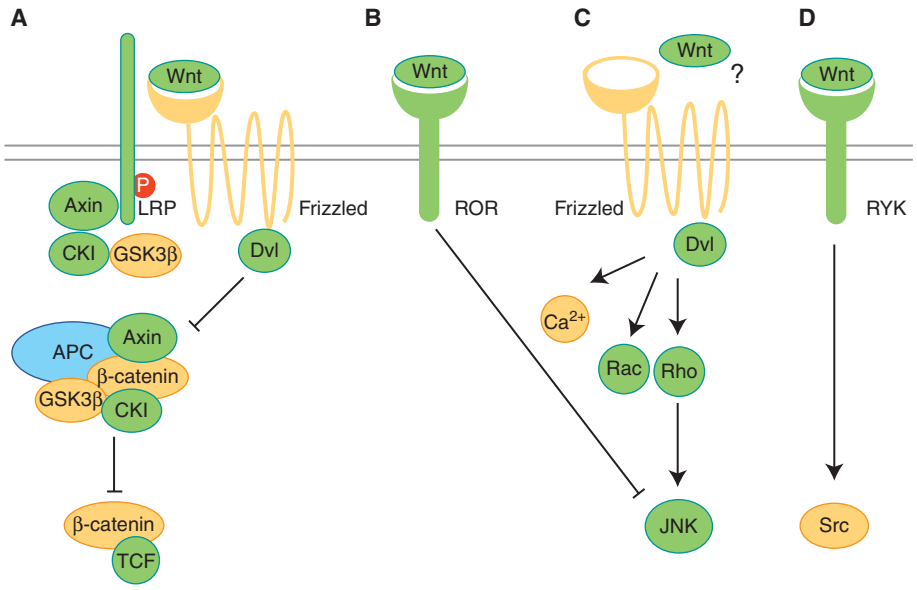

Figure 1. Evolution of Wnt pathways. Proteins involved in different Wnt pathways (*A–D*) are shown and labeled according to their occurrence in protozoans (yellow), pre-bilaterians (green), and bilaterians (blue). (*A*) Binding of Wnt to Frizzled receptor and LRP activates β-catenin/TCF signaling. The β-catenin destruction complex, including APC, Axin, GSK3β, and CKI, sequesters and phosphorylates β-catenin. Phosphorylation of LRP is crucial for Wnt/β-catenin signaling. (*B*) Binding of Wnt to the receptor tyrosine kinase (RTK) Ror2 activates Jnk and inhibits β-catenin/TCF signaling. (*C*) Frizzled receptors in planar cell polarity (PCP) of vertebrates act via downstream messengers including Dishevelled (Dvl), small Rho GTPases, Jnk, or Ca^{2+}. In flies, PCP can be activated independently of Wnt-Frizzled binding (?). (*D*) Binding of Wnt proteins to RYK RTKs results in the activation of Src proteins. (Adapted from van Amerongen and Nusse 2009; reprinted, with permission, from the authors.)

(Grimson et al. 2000; Coates et al. 2002), but other components of the Wnt signal transduction cascade, including Dsh, Axin, and APC proteins, have not been identified (Harwood 2008). A GSK3 ortholog (GSKA) acts in cell growth and differentiation processes of *Dictyostelium* (Harwood 2008), for example, in the homeostasis of prestalk and prespore cells (Harwood et al. 1995; Schilde et al. 2004). Although GSK3 was also identified in other eukaryotes (Srivastava et al. 2008), the Axin-binding site present in GSK3 and other non-metazoan GSK3-related kinases can be considered as a preadaptation for the function of GSK3 in Wnt signaling.

The most notable member of the Wnt pathway present in *Dictyostelium* is the β-catenin-related molecule Aardvark (Aar) (Grimson et al. 2000). Aardvark is essential for the cAMP-mediated induction of pre-spore cells, where it acts together with GSKA. However, in contrast to the negative regulation of canonical Wnt signaling by GSK3, that is, phosphorylation of β-catenin by GSK3, *Dictyostelium* GSKA acts positively on Aar to induce pspA expression (Harwood 2008). As pointed out by Harwood (2008), this mechanism is reminiscent to the regulation of noncanonical *Wnt*s in *C. elegans* (Hardin and King 2008).

Recently, an α-catenin ortholog has been discovered that, together with the β-catenin ortholog Aar, plays a role in cell adhesion (Dickinson et al. 2011). Mutations of *Aar* result in a loss of cell junctions (Coates and Harwood 2001; Hardin and King 2008). This is remarkable because cadherin-like molecules have not been found in *Dictyostelium* and were only identified in the genome of the choanoflagellate

Monosiga brevicollis (Abedin and King 2008). This could indicate that β-catenin has an ancient function in cell adhesion that predates the evolution of its function in Wnt signaling (Dickinson et al. 2011).

Wnt Transcription Factors and Target Genes

No orthologs to the TCF/Lef transcription factors have been described outside metazoans, suggesting that these are metazoan-specific innovations (Srivastava et al. 2010). However, a *Brachyury*-related T-box gene was found in *Capsaspora owczarzaki*, a protist that is closely related to choanoflagellates and metazoans (Sebe-Pedros et al. 2011). Brachyury is a direct downstream target of Wnt signaling during posterior growth of vertebrates (Yamaguchi et al. 1999), where it directly regulates Wnt signaling (Martin and Kimelman 2008, 2009). At which point in animal evolution this positive Brachyury–Wnt loop was established is unknown so far (Martin and Kimelman 2008), but the coexpression of *Wnt* and *Brachyury* in basal metazoans (Technau et al. 2000) suggests that it was established during early metazoans evolution.

ORIGIN OF DIFFERENT WNT PATHWAYS IN PRE-BILATERIANS

Wnt signaling controls a variety of different cellular behaviors including cell proliferation, stem cell maintenance and differentiation, coordinated cell movement, and the establishment of tissue polarity (Croce and McClay 2008; van Amerongen and Nusse 2009). Based on their ability to induce an ectopic axis in *Xenopus* embryos (McMahon and Moon 1989) or planar cell polarity and convergent extension movements (Heisenberg et al. 2000), the different Wnts have frequently been classified as "canonical" or "noncanonical" Wnts. However, this is an oversimplification because how different Wnts signal is also determined by the receptors with which they interact. For example, the "noncanonical" Wnt5a can also signal in the canonical β-catenin pathway, if it is exposed to the right receptor on the cell, that is, Fz4 (van Amerongen et al. 2008; van Amerongen and Nusse 2009). As shown in Figure 1, in canonical β-catenin signaling, Wnts can bind to Frizzled and LRP and thereby activate β-catenin/TCF. Wnts can also interact in a β-catenin-independent manner with RYK receptors in Src signaling or with ROR in JNK signaling (via small Rho GTPases and c-Jun amino-terminal kinase) (van Amerongen and Nusse 2009). In some cases, multiple Wnts are required to induce a specific cellular response (van Amerongen and Nusse 2009).

It is difficult to judge which of the Wnt functions might represent the ancestral form—the β-catenin dependent or independent—or how this might be related to the specific structure of Wnt ligands. There are hints that canonical and noncanonical functions of Wnt proteins may be conserved during evolution (Rigo-Watermeier et al. 2011), but the distinction between both groups of ligands is not sharp (see above). It could therefore easily be that the first Wnt ligands had multiple functions in different pathways. Because the repertoire of Wnt receptors was smaller in basal metazoans compared with higher bilaterians, it is tempting to speculate that Wnt receptors in basal metazoans are more promiscuous than in vertebrates and that the evolution of Wnt receptors resulted in a larger and more refined Wnt-receptor repertoire.

Considering the core function of β-catenin in cell adhesion, an ancient role of β-catenin in cell adhesion might have further evolved to control local changes in cell signaling by Wnt ligands by creating a collective of cells with similar signaling properties (Fig. 2). These positional cues were probably the origin of the canonical pathway, fostered by the evolution of multidomain scaffolding proteins.

Porifera, Placozoa, and Ctenophora

Porifera (sponges), Placozoa, and Ctenophora (comb jellies) are simple metazoans for which a basal set of Wnt ligands, receptors, and cytoplasmic transducers has been identified. Their *Wnt* gene repertoire is less complex than that of any other metazoans. Although the phylogenetic position of placozoans and ctenophores is still under debate (Ruiz-Trillo et al. 2008), it is

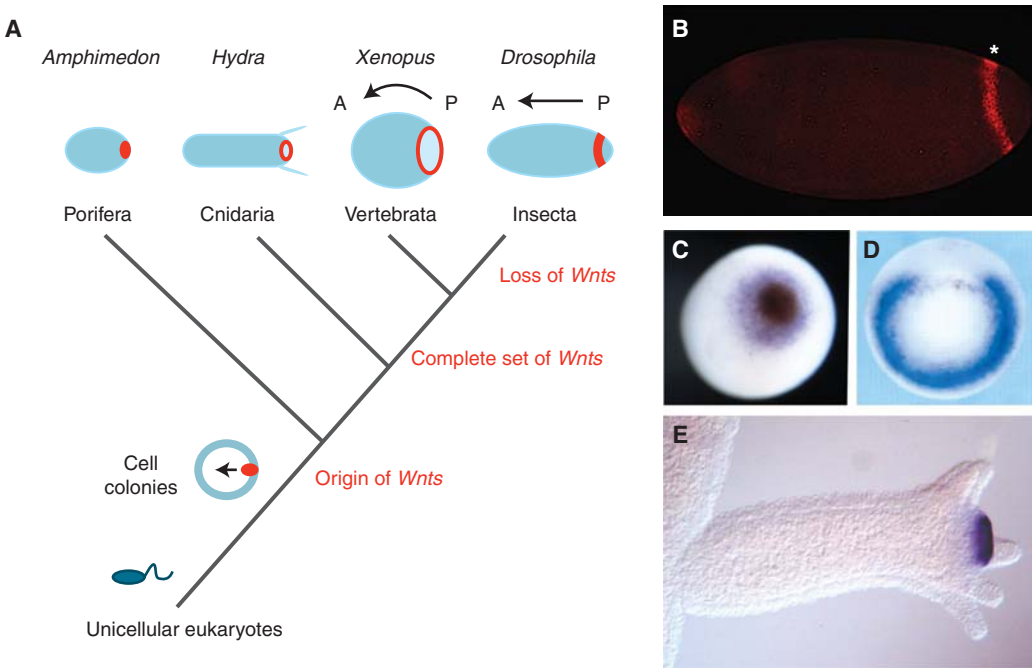

Figure 2. Evolution of Wnt signaling and axis formation during metazoan evolution. (*A*) Wnt signaling centers (red) evolved at the transition from unicellular (e.g., *Monosiga*) to multicellular eukaryotes. In all studied metazoans (sponges, cnidarians, deuterostomes, and protostomes), a posterior Wnt signaling center defines the posterior pole of the body axis. The number of *Wnt* gene subfamilies increased from the first metazoans to cnidarians, whose complete set of *Wnt* genes is only retained in basal deuterostomes and protostomes. Protostomes are characterized by frequent loss of *Wnt* gene subfamilies. (*B*) The posterior *Wg* (*Wnt1*) stripe in *Drosophila.* (Figure kindly provided by Drs. P. Vorwald and E. De Robertis, Howard Hughes Medical Institute, UC Los Angeles.) (*C*) *Wnt* expression at the blastopore of a *Amphimedon* gastrulae (*AmqWntA*), at the blastopore of an early *Xenopus* gastrulae (*Wnt8*) (figure kindly provided by Dr. Maja Adamska, SARS Center, Bergen), and (*D*) and (*E*) at the hypostome of *Hydra* polyps (*Wnt3*). (Fig. 2D from Steiner et al. 2006; reprinted, with permission, from The Company of Biologists © 2006; Fig. 2E from Hobmayer et al. 2000; reprinted, with permission, from the author.)

likely that they represent a metazoan clade that branched off early in metazoan evolution.

Placozoans, disc-shaped creeping creatures with unknown embryology, have two epithelial layers lacking any gastric cavity (Srivastava et al. 2008). The genome contains 11,514 protein-coding genes that are closely related to those of cnidarians (Srivastava et al. 2008). The only known species, *Trichoplax adhaerens*, has three unclassified *Wnt* genes and major components of canonical Wnt signaling (Dsh, Frz, GSK3, Axin, β-catenin TCF), but no Wnt antagonists like Dkk or secreted Frizzled-related proteins (Srivastava et al. 2008). The expression patterns of the *Wnt* genes and β-catenin are unknown.

Sponges have a simple body plan that develops from a blastula, producing a tube-shaped diploblastic larva that is similar to the planula larva of cnidarians. This diploblastic tube invaginates at multiple positions and develops a branched filtering channel system (Brusca and Brusca 2002). The filtering cells (choanocytes) are similar in morphology to the protozoan choanoflagellate *Monosiga* (King et al. 2008). The genome of the demosponge *Amphimedon queenslandica* shows significant conservation of gene families with those of cnidarians and bilaterians (Srivastava et al. 2008). Besides β-catenin, Fzd, and GSK3, the main components of the canonical Wnt/β-catenin pathway

(SFRP, Lrp5/6, Dvl, Axin, APC, TCF, and Groucho) are present, but those of the noncanonical pathways are missing (Adamska et al. 2007, 2010). *Amphimedon* contains three, and the homoscleromorph sponge *Oscarella* two, *Wnt* genes, which have proven difficult to classify (Lapebie et al. 2009; Adamska et al. 2010; Srivastava et al. 2010). *WntA* expression is restricted to the posterior pole of the *Amphimedon* larva, which is also marked by a pigment ring (Fig. 2C). In *Oscarella*, *Wnt-I* is also expressed at the canal openings (ostia), and β-catenin activation induces ectopic ostia (Lapebie et al. 2009). This is reminiscent of the blastoporal organizer in cnidarians (Windsor and Leys 2010), and future work must reveal to what extent these *Wnt* genes also contribute to noncanonical Wnt function.

In the ctenophore *Mnemiopsis leidyi*, orthologs of the main components of Wnt signaling have been found (β-catenin, Dishevelled, 2Fz, sFrp, TCF, Pygopus, Porcupine, LRP5/6, GSK3, APC, CK1, Groucho, Wntless), including four Wnt ligands (WntA, Wnt6, Wnt9, Wntx) (Pang et al. 2010). β-catenin is expressed at the oral pole during gastrulation, whereas the four Wnt ligands are expressed only late at the aboral side of the larva, when tentacles and sensory organs form (Pang et al. 2010).

Cnidaria

Cnidarians show an archetypal gastrula-shaped body plan. The genomes of the sea anemone *Nematostella* (Putnam et al. 2007) and the freshwater polyp *Hydra* (Chapman et al. 2010) reveal a gene repertoire with about 18,000 bona fide protein-coding genes, illustrating the high genomic complexity of the common bilaterian–cnidarian ancestor (Putnam et al. 2007; Chapman et al. 2010). All bilaterian *Wnt* gene subfamilies are present in these cnidarian genomes (Kusserow et al. 2005; Lee et al. 2006; Lengfeld et al. 2009). In *Nematostella*, *Wnt-9* has not been found (Kusserow et al. 2005; Lee et al. 2006), whereas in *Hydra*, *Wnt4*, *-6*, and *-A* are missing. Three *Hydra Wnt* genes were classified as *HyWnt9/10-a*, *-b*, and *-c* (Lengfeld et al. 2009). This completeness of *Wnt* gene subfamilies in cnidarians suggests that the common (eumetazoan) ancestor of cnidarians and bilaterians already possessed a complete repertoire of *Wnt* gene ligands (Kusserow et al. 2005).

Cnidarian Wnts act in the canonical and the PCP pathways. All core components of the Wnt receptor and the β-catenin destruction complex are present. The number of Frizzled (Fzd) receptors is lower than that of the ligands (four Fzd in *Hydra*, and six in *Nematostella*), suggesting that the radiation of *Wnt* genes was followed by diversification of the receptors. Similarly to sponges, *Nematostella* Axin is lacking a clear β-catenin interaction domain, and APC shows only clear Armadillo repeats and a PDZ-domain (Adamska et al. 2010), raising questions as to whether the assembly of the cnidarian β-catenin destruction complex is as complete as in bilaterians. Orthologs of PCP signaling like Strabismus/Van Gogh, RhoA, Rock-2, and RAC1 are present, and all secreted Wnt antagonists have been identified, that is, sFrp, Wnt inhibitory factor (WIF), Cerberus, and Dkk proteins (Augustin et al. 2006; Guder et al. 2006a; Lee et al. 2006; Holstein et al. 2011; Technau and Steele 2011). Components of Ca^{2+} signaling (PLC, PKC, CaMKII, and Calcineurin) are also present, but no functional data exist on the putative role of Wnt5 or any other cnidarian Wnt ligand in Ca^{2+} signaling.

Wnt/β-catenin signaling has a major function in gastrulation and anterior–posterior patterning throughout metazoan development, because most *Wnt* genes are expressed at the site of the blastopore (Fig. 2E). During gastrulation, β-catenin is activated at the site of the future blastopore, and blocking GSK3 causes extended gastrulation movements in *Nematostella* (Wikramanayake et al. 2003). Dsh is necessary for this blastoporal activation (Lee et al. 2007). In the hydrozoan *Clytia*, Fz1 and maternal Wnt3 are required for oral β-catenin activation (Momose and Houliston 2007; Momose et al. 2008). In *Nematostella* gastrulation and planula larvae, *Wnt*s are expressed in overlapping domains along the oral–aboral axis (Kusserow et al. 2005).

Most *Wnt* genes are also expressed in a similar cascade during head regeneration and

budding of *Hydra* (Lengfeld et al. 2009). Here, canonical Wnt signaling acts upstream of noncanonical Wnt signaling, which is required for mass-tissue movements during tissue evaginations. Noncanonical processes are mediated by *Wnt5*, *Wnt8*, *Fzd2*, and *Dsh* together with JNK (Philipp et al. 2009). A role for *Nematostella* Wnts in *Xenopus* Wnt-5a/Ror2 and Wnt-11 PCP signaling was shown by different morphant phenotypes that differ in PAPC regulation, cell polarization, cell protrusion formation, and microtubule orientation (Rigo-Watermeier et al. 2011). NvWnt-5 rescued XWnt-11 and NvWnt-11 rescued XWnt-5a, suggesting that specific structures in Wnt ligands are important for receptor complex recognition in Wnt signaling.

PATTERNS OF GENE GAIN AND LOSS IN BILATERIANS

An intriguing feature in metazoan evolution is the frequent loss of *Wnt* gene subfamilies in protostomes (Table 1). All protostome lineages lack the *Wnt3* ortholog, whereas *Wnt1*, *Wnt5*, and *WntA* are well conserved in most protostomes. The other *Wnt* gene subfamilies have been significantly reduced in various groups (Table 1). Gene loss of *Wnt*s is also evident in some deuterostomes, but to a much lesser extent.

Lophotrochozoa

The lophotrochozoans were identified as a monophyletic group by molecular phylogenies (Halanych et al. 1995) and constitute forms with spiral cleavage and a trochophora larva (annelids and mollusks) and flatworms. Twelve Wnt subfamilies have been reported for annelids (*Capitella teleta* and *Platynereis dumerlii*), a major lophotrochozoan group. This indicates that basal protostomes also had an almost complete set of *Wnt* genes (Cho et al. 2010). Other annelids and mollusks have lost several *Wnt* gene subfamilies, that is, *Wnt8*, *-9*, and *-A* in *Helobdella robusta* or *Wnt4*, *-5*, *-6*, *-7*, *-8*, *-9*, *-11*, and *-16* in *Patella vulgata* (Prud'homme et al. 2002; Cho et al. 2010; Janssen et al. 2010; Riddiford and Olson 2011). The *Wnt* genes of *P. dumerilii* show blastoporal and posterior expression in the growth zone (segment addition zone) of trochophoran larvae, and at the posterior side of each segment (Janssen et al. 2010), reminiscent to arthropods (see below).

It has been suggested that ciliated acoel and turbellarian flatworms are the most ancient bilaterians (Ruiz-Trillo et al. 1999; Baguna and Riutort 2004). Their shape is similar to cnidarian planulae, but they have an elaborated gut with one pharyngeal opening derived from the blastopore. A survey of *Wnt* genes in platyhelminths, including *Schmidtea mediterranea* (turbellarian planarian), the parasitic *Schistosoma mansoni* (cestode), *Echinococcus granulosis* (trematode), and *Hymenolepis microstoma* (trematode) revealed a strong tendency toward gene loss (Riddiford and Olson 2011). Planarians possess nine *Wnt* genes, classified as members of the *Wnt1*, *-2*, *-5*, and *-11* subfamilies, with putative gene duplications in the *Wnt11* group (Gurley et al. 2010; Riddiford and Olson 2011). Because most platyhelminths are highly diverged, it will be essential for future studies to unravel the *Wnt* gene repertoire of acoel flatworms, the putative bilaterian stem group.

In *S. mediterranea*, all *Wnt* genes are predominantly expressed with sFrp genes in discrete, complex, overlapping domains along the A/P axis, in a manner that suggests a possible steady-state posterior-to-anterior gradient of β-catenin activity (Gurley et al. 2008, 2010; Petersen and Reddien 2008). This is reminiscent of the overlapping *Wnt* expression patterns observed in the radially symmetric cnidarian body plan, the main axis of which is determined by β-catenin signaling (Gurley et al. 2010). Wnt and sFrp genes play an important role in flatworm regeneration (Reddien and Sanchez Alvarado 2004; Gurley et al. 2008, 2010; Petersen and Reddien 2008; Iglesias et al. 2011). Down-regulation of β-catenin in regenerating planarians produces an enhancement of anterior head structures (Gurley et al. 2008; Iglesias et al. 2008; Petersen and Reddien 2008). Inhibition of APC or silencing of Axin results in the regeneration of tails from anterior-facing wounds (Gurley et al. 2008) or in two-tailed planarians without a brain (Iglesias et al. 2008). In line with these data, inhibition of the Wnt inhibitor

Table 1. Distribution of metazoan *Wnt* genes

		N	Wnt1	Wnt2	Wnt3	Wnt4	Wnt5	Wnt6	Wnt7	Wnt8	Wnt9	Wnt10	Wnt11	Wnt16	WntA	n.d.
	Choanoflagellata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Radiata	Placozoa	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	Porifera	2	0	0	0	0	0	0	0	0	0	1(0)	1(2)	1(0)	0	0
	Ctenophora	1	0	0	0	0	0	1	0	0	1	1	0	0	1	0
	Cnidaria	2	1	1	1	1 (0)	1	1	1	1 (2)	0	1 (3)	1	1	1	0
Lophotrocho-zoa	Mollusca	2	1	2	0	1 (0)	1 (0)	1 (0)	1 (0)	0	1 (0)	1	1 (0)	1 (0)	1	0
	Annelida	3	1	1	0	1	1 (2)	1	1	1 (0)	1 (0)	1	1 (3)	1 (2)	1 (0)	0
	Platyhelminthes	4	1	1	0	1 (3)	1	0	0	0	0	0	1 (2)	0	0	0
Ecdysozoa	Nematoda	1	0	0	0	1	1	0	0	0	1	1	0	0	1	0
	Chelicerata	2	1	1	0	1	1	1	1 (2)	1	1	0	1 (2)	1	1 (0)	0
	Crustacea/Myriapoda	2	1	1	0	1	1	1	1	1	1	1	1	1	1	0
	Insecta	6	1	0	0	0	1	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	0
Deuterostomia	Echinodermata	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0
	Hemichordata	1*	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Urochordata	1	1	1	1	2	1	1	2	0	1	1	0	1	0	0
	Cephalochordata	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0
	Vertebrata	>10	1 (2)	2 (5)	2 (3)	1 (2)	2	1 (2)	2 (3)	2 (3)	1 (3)	2 (3)	1 (3)	1	0	0

Notes: N indicates the number of species analyzed in a clade; numbers in parentheses indicate deviations of the number of Wnt orthologs found in species of a given clade. Colors in filled boxes indicate the abundance of *Wnt* gene subfamily members present in a given clade (gene duplication or gene loss). Note the loss of *Wnt3* in protostomes and the loss of *WntA* in chordates. Data are based on molecular Wnt phylogenies by Kusserow et al. (2005), Lee et al. (2006), Croce and McClay (2008), Srivastava et al. (2008), Lengfeld et al. (2009), Lapebie et al (2009), Adamska et al (2010), Gurley et al. (2010), Janssen et al (2010), and Riddiford and Olson (2011).

(*) The Wnts from the hemichordate *Saccoglossus kowalevskii* correspond to Gl: 259013321 (*Wnt1*), Gl: 259013323 (*Wnt2*), Gl: 259013327 (*Wnt3*), Gl: 291234645 (*Wnt4*), Gl: 269785077 (*Wnt5*), Gl: 268054405 (*Wnt6*), Gl: 269785051 (*Wnt7*), Gl: 269785053 (*Wnt8*), Gl: 269785055 (*Wnt9*), Gl: 291244845 (*Wnt10*), Gl: 269785075 (*Wnt11*), Gl: 197320537 (*Wnt16*), Gl: 284005183 (*WntA*).

notum induces the regeneration of anterior-facing tails instead of a head, and double-RNAi experiments indicate that *notum* inhibits Wnt signaling in a feedback inhibition to promote head regeneration (Petersen and Reddien 2011). Thus, Wnt/β-catenin signaling clearly controls the tail-versus-head (i.e., posterior–anterior) axis in intact and regenerating planarians.

Ecdysozoa

Gene loss has also occurred in various ecdysozoan groups (Table 1), the most prominent having occurred in *Caenorhabditis elegans* (nematodes), which is lacking eight *Wnt* genes subfamilies (*Wnt1–3, Wnt6–8, Wnt11*, and *Wnt16*). The other major ecdysozoan groups are arthropods. Basal arthropods show a rich *Wnt* gene repertoire, indicating that the common ancestor of arthropods only lacked *Wnt-3* (Janssen et al. 2010). Members of 12 *Wnt* gene subfamilies were characterized at the transcriptional level (Janssen et al. 2010). A relatively complete set of protostome *Wnt* genes was found in the genomes of the crustacean *Daphnia pulex* (12 *Wnt* subfamilies), and in chelicerates and myriapods, where *Wnt10* has been lost (Janssen et al. 2010). In comparison, insects show significant gene loss (Table 1). Only *Wnt1* (wingless) and *Wnt5* are present in all insects investigated so far. The other *Wnt* genes are absent in several insects, and there seems to be a tendency for increasing loss of *Wnt* genes, for example, *Wnt2* and *Wnt4* are lacking in all insects, *Wnt16* in all holometabolous insects, *Wnt11* in all dipterans, and *WntA* in *Drosophila* (Janssen et al. 2010).

The expression patterns of arthropod *Wnt* genes reveal that most overlap in a segment addition or growth zone, and in segmentally reiterated patterns defining the parasegments (Janssen et al. 2010).

These expression patterns are remarkably similar to annelids', suggesting similar Wnt regulation mechanisms during segment and parasegment formation in annelids and arthropods, respectively (Janssen et al. 2010). *Wnt1/Wg* is a prototypical *Wnt* gene for the posterior growth zone in annelids and arthropods. It is notable that even in *Drosophila* it shows an early posterior expression domain (Fig. 2B). In *Drosophila*, all segments are formed simultaneously (long germ band formation) and the posterior band of *Wg* is expressed in the early blastoderm before the segment-polarity stripes of *Wg* are formed (Vorwald-Denholtz and De Robertis 2011). This posterior *Wg* band might represent a remnant of a posterior signaling center (Vorwald-Denholtz and De Robertis 2011).

Of particular interest is the fate of *Wnt8* in arthropods. In the spider *Achaearanea* sp. Wnt8 is required for posterior development and the maintenance of the growth zone (McGregor et al. 2008). It is expressed in a solid circular domain at the posterior end of a radial symmetric embryo before its transition to axial symmetry (Fig. 1A in McGregor et al. 2008). In *Drosophila*, Wnt8 has a completely different function and acts as a feedback inhibitor of the NF-κB homolog Dorsal (the gene was therefore named *Wnt inhibitor of Dorsal*, *WntD*) in DV signaling and immunity (Gordon et al. 2005; Gordon and Nusse 2006). *WntD* expression is under the control of Toll/Dorsal signaling, and increased levels of WntD block the nuclear accumulation of Dorsal. WntD signals independently of the β-catenin homolog Armadillo (Ganguly et al. 2005; Gordon et al. 2005).

Deuterostomia

In deuterostomes, data from more than 10 completely sequenced vertebrate genomes show unambiguously that all *Wnt* gene subfamilies except *WntA* were present at the base of vertebrate radiation (Table 1). Because *WntA* was identified in the genome of echinoderms (Sodergren et al. 2006; Croce and McClay 2008), the common ancestor of deuterostomes must have had a complete set of *Wnt* genes. Genome data suggest some Wnt gene loss in the cephalochordate amphioxus (*Wnt2*, *-9*, *-16*, and *-A*) (Putnam et al. 2007) and the urochordate ascidian *Ciona* (*Wnt8* and *-11*) (Dehal et al. 2002). According to NCBI annotations, the only deuterostome with a complete set of *Wnt* gene subfamilies is the hemichordate *Saccoglossus kowalevskii* (Table 1). Although the *Saccoglossus* data must be confirmed by a specific phylogenetic study, it

is likely that the deuterostome radiation started with a complete set of *Wnt* gene subfamilies.

The function and localization of Wnt and β-catenin signaling in deuterostome AP-axis formation has been recently reviewed in detail (De Robertis 2010; Niehrs 2010; Holstein et al. 2011). The general picture that emerges is that β-catenin plays an important function in the early polarization of the animal–vegetal axis of the embryo (Martin and Kimelman 2009). Similar to pre-bilaterian animals, β-catenin is vegetally localized in many basal deuterostomes. In sea urchins (echinoderms), development starts with clear polarity from the animal to the vegetal pole that resembles the oral–aboral body axis in cnidarians. β-catenin signaling marks the vegetal pole of the sea urchin embryo (Wikramanayake et al. 1998, 2004; Logan et al. 1999) and is required for the formation of the endoderm and mesoderm. Overactivation of β-catenin with LiCl results in vegetalized embryos with ectopic endoderm (Darras et al. 2011). Furthermore, in many chordates, a peak expression of β-catenin at the vegetal and blastoporal side defines a gradient along the AP axis. In both ascidians and amphioxus, nuclear-localized β-catenin marks the blastopore, which, in turn, defines the future posterior end of the embryo (Imai et al. 2000). Activation of β-catenin signaling by LiCl posteriorizes the amphioxus embryo, which is evident by loss of the neural plate (Yu et al. 2007; Onai et al. 2009). Similarly to the posterior growth zone in amphioxus tail buds (Schubert et al. 2001), nuclear β-catenin also forms a posterior-to-anterior gradient in vertebrates. Wnt signaling is involved in tail development in zebrafish (Agathon et al. 2003; Shimizu et al. 2005; Thorpe et al. 2005), where Wnt3a determines the anterior–posterior position of the somite determination front (Aulehla et al. 2003; Dunty et al. 2008; De Robertis 2010; Niehrs 2010).

SYNTENY AND EVOLUTION OF *WNT* GENES

Wnt genes show intriguing axial expression patterns along the anterior–posterior axis in cnidarians (e.g., in *Nematostella*). However, this is also true in many other bilaterians with complete sets of Wnts, where expression during embryogenesis or regeneration indicates an underlying order in transcriptional regulation. The pattern of overlapping Wnt ligands that is used in cell fate specification and patterning was characterized as the "Wnt code" (Guder et al. 2006a) or the "Wnt landscape" (Janssen et al. 2010).

Although *Wnt* genes do not show a proper Hox-type collinearity on the genomic level (Sullivan et al. 2007), there is a certain degree of *Wnt* clustering and synteny (see also Fig. 3). A primordial cluster of *Wnt* genes (*Wnt1*, *-6*, and *-10*) was first postulated by Nusse (2001) for the *Drosophila* and human genomes. Accordingly, the human genome contains duplicated copies of this cluster, but *Wnt6* was deleted from 12q13 and *Wnt1* was deleted from 2q35 (Fig. 3). The clustering of *Wnt1*, *-6*, and *-9/10* in a molecular phylogeny with *Wnt*s from *Nematostella* (Kusserow et al. 2005) and *Hydra* (Lengfeld et al. 2009) supports this hypothesis. A second conserved cluster was found for *Wnt5* and *Wnt7* (Sullivan et al. 2007). New data on the arrangement of *Wnt* genes in the *Daphnia* genome revealed two syntenic clusters of these genes: *Wnt9–Wnt1–Wnt6–Wnt10* and *Wnt5–Wnt7* (Janssen et al. 2010). *Lottia gigantea* shows a similar organization of these *Wnt* genes and therefore reflects the ancient cluster of *Wnt* genes in eumetazoans (Nusse 2001; Sullivan et al. 2007; Cho et al. 2010; Janssen et al. 2010). These two *Wnt* clusters are also supported by recent molecular phylogenies on *Wnt* genes (Cho et al. 2010; Janssen et al. 2010). How the interacting Wnt ligands are regulated is rather unknown so far, but it is tempting to speculate that there is a regulative hierarchy of *Wnt* gene expression similar to that described for *Hydra* (Nakamura et al. 2011) or *Drosophila* (van de Wetering et al. 1997; Lessing and Nusse 1998).

THE ORGANIZER: EVOLUTION OF THE POSTERIOR WNT SIGNALING CENTER

Cnidarians are the only pre-bilaterian animals with a complete set of *Wnt* genes and a blastoporal signaling center that is similar to that in deuterostomes and protostomes. This signaling center has been investigated in detail in the

freshwater polyp *Hydra*, where it was shown to exhibit inductive properties that are reminiscent of the classical vertebrate organizers.

The *Hydra* Organizer

The *Hydra* organizer corresponds to the oral end of an adult polyp, which has retained the signaling properties of the blastoporal signaling center. It is known as the *Hydra* "head" organizer. The naming is misleading because the *Hydra* "head," being a blastoporal region, represents the posterior pole of the body axis, and the *Hydra* "foot" is therefore the anterior pole (Meinhardt 2002; Niehrs 2010; Holstein et al. 2011). We therefore name it the "*Hydra* Organizer (HO)." A group of 10–50 ectodermal and endodermal epithelial cells constitutes this signaling center (Technau et al. 2000), and when transplanted to an ectopic site, the HO induces a secondary body axis by recruiting host tissue (Browne 1909; Broun and Bode 2002).

Short- and Long-Range Wnt Signaling

Activation of Wnt signaling stimulates the expression of all *Wnt* genes as well as of the Wnt target genes *Tcf* and *Brachyury*, resulting in numerous spot-like *Wnt* expression domains along the body column (Broun et al. 2005; Guder et al. 2006b; Nakamura et al. 2011). It is generally accepted that each of these Wnt signaling centers creates a gradient of diffusible Wnt, although thus far the distribution of none of the cnidarian Wnts has been visualized by using either antibodies or reporter constructs.

The parameters of the HO were determined experimentally under conditions of de novo pattern formation (Technau et al. 2000) in reaggregates from dissociated single-cell suspensions (Gierer et al. 1972). Clusters with a minimal size of five to 15 epithelial Wnt3-expressing cells are necessary and sufficient to form de novo a HO in an aggregate, generating a lateral inhibition with a diffusion range of up to 1000 μm (Technau et al. 2000).

These parameters fit with the reaction-diffusion model of *Hydra* pattern formation, assuming short-range autocatalytic activation and long-range inhibition (Gierer and Meinhardt 1972). In transgenic *Hydra*, an activator element of the *Hydra Wnt3* promoter was identified that maintains *Wnt3* transcription in the HO by recruiting TCF and β-catenin (Nakamura et al. 2011).

The molecular nature of long-range inhibition could also be explained by Wnt secretion.

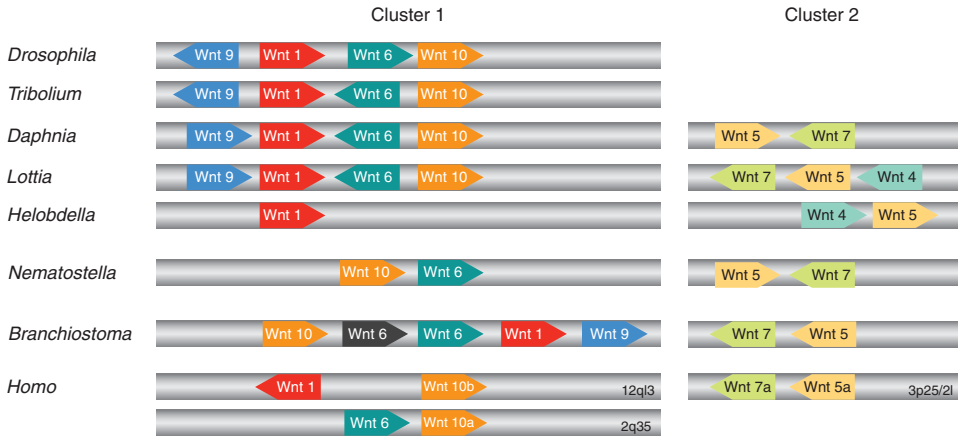

Figure 3. Synteny of metazoan *Wnt* genes. The relative position and orientation of *Wnt* genes with common scaffolds/chromosomes are shown for *D. melanogaster, Tribolium castaneum, D. pulex, L. gigantea, H. robusta, Nematostella vectensis, Branchiostoma floridae*, and *Homo sapiens* (not to scale). Numbers indicate specific chromosomes in the case of *H. sapiens*. (Figure created from data from Nusse [2001], Sullivan et al. [2007], Cho et al. [2010], and Janssen et al. [2010].) Note that there are more Wnts for a given species that are not clustered (see Table 1).

According to a model proposed by Bartscherer and Boutros (2008), Wnt ligands may have different diffusion ranges related to their mode of secretion into different vesicle populations (Bartscherer and Boutros 2008; Lorenowicz and Korswagen 2009). Wnt can be secreted with a short diffusion range at the apical side of an epithelial layer, whereas Wnt proteins that are associated with lipoprotein particles result in a longer range. We presume that both modes of Wnt secretion contribute to short- and long-range signaling in *Hydra* (Fig. 4B). Because β-catenin and Wnt3 act in an autoregulatory loop, a gradient of Wnt3 will result in a graded distribution of nuclear β-catenin along the body column (Guder et al. 2006a). A long-range Wnt3 signal can activate both a transcriptional repressor (see below) and the Wnt antagonist

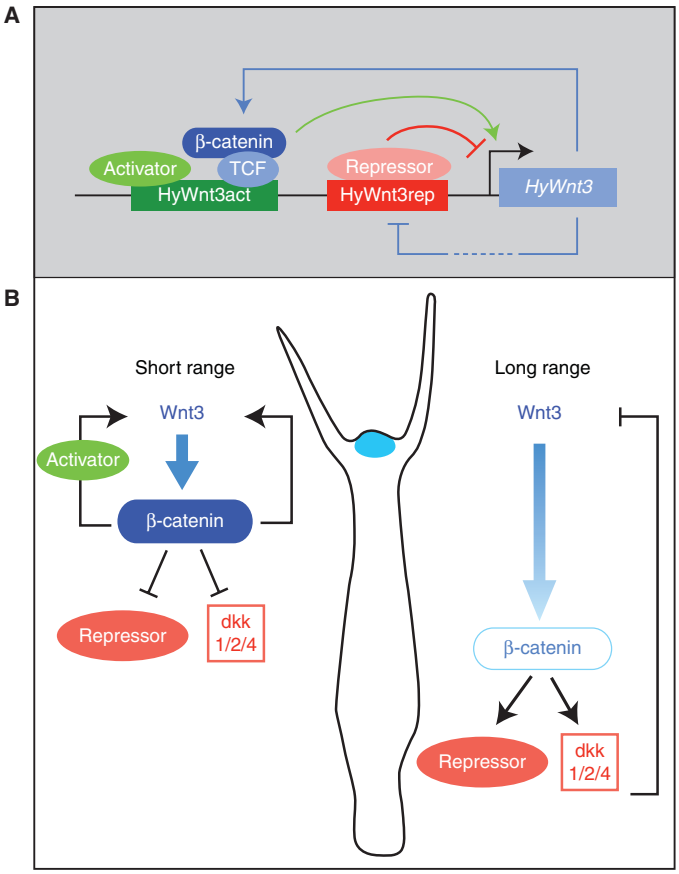

Figure 4. Transcriptional control of Wnt signaling in *Hydra*. (*A*) Model showing the transcriptional regulation of head organizer-specific *HyWnt3* expression. *HyWnt3* expression is controlled by two distinct *cis*-regulatory elements; the activator (HyWnt3act, green) and the repressor (HyWnt3rep, red) are positively and negatively regulated by Wnt/β-catenin signaling, respectively. The β-catenin/TCF complex and putative activators (light green) bind to HyWnt3act, and their combinatorial inputs act in *HyWnt3* transcription (green arrow). Potential repressors (red) bind to a repressor element (red) and inhibit *HyWnt3* expression. (Figure adapted from Nakamura et al. 2011; reprinted, with permission, from the author.) (*B*) Presumed distribution or activity of β-catenin depends on the diffusion range of Wnt3. Following short-range diffusion, the concentrations of Wnt3 and β-catenin are high, thereby repressing putative repressors and dkk1/2/4. Following long-range diffusion, the level of nuclear β-catenin is lower, inducing putative repressors including dkk1/2/4. Positive and negative regulation restricts *HyWnt3* expression (blue) to the head organizer region.

Dkk1/2/4 in the body column (Fig. 4B). Fields of lateral inhibition can be visualized by the suppression of Dkk1/2/4 expression in *Hydra* polyps after activation of Wnt signaling by using alsterpaullone treatment (Guder et al. 2006a,b). The double function of Wnt3 as a short-range activator and long-range inhibitor was recently proposed and elaborated in a model by Meinhardt (2012).

An alternative explanation for the long-range signaling could be that a cascade of sequential *Wnt* gene activation and expression could explain the "field" behavior of Wnt signaling, thereby extending its range. The cascade of consecutive *Wnt* activation during gastrulation in *Nematostella* (Kusserow et al. 2005) and during regeneration and bud formation in *Hydra* (Lengfeld et al. 2009) would support this alternative model of long-range Wnt signaling.

Local Restriction of the Organizer

In line with these new hypotheses, we found that Wnt signaling can also be effectively suppressed at the transcriptional level (Nakamura et al. 2011). The removal of a repressor element in the regulatory region of Wnt3 resulted in an expansion of the Wnt3 gene expression domain toward the gastric region (Fig. 5) (Nakamura et al. 2011). This shows that transcriptional regulation is essential to restrict Wnt expression to the site of the signaling center. Transcriptional regulation of *Wnt3* expression is probably at the core of Wnt/β-catenin signaling in *Hydra*, and only complemented by Dkk1/2/4. The creation of local sources for the secretion of Wnt ligands might have been an important step in the evolution of Wnt signaling centers. We propose that *cis*-regulatory control mechanisms combining short-range autoactivation and long-range repression resulted in a locally defined Wnt signaling center. This signaling center may be independent of the function of extracellular Wnt antagonists, which could explain why Wnt antagonists are absent from several metazoan lineages (Nakamura et al. 2011).

CONCLUDING REMARKS

The broad variety in animal forms appears to be founded on astonishingly few signaling pathways that are shared by all major metazoan phyla. The Wnt signaling pathways had a critical function in setting up an ancient blastoporal signaling center. Molecular components of this signaling network can only be traced in part to pre-metazoan origins, indicating that major novelties were linked to the emergence of the metazoan stem. A core set of *Wnt* genes was already present in sponges, but only in cnidarians is a complete functional repertoire of *Wnt* ligands present. All *Wnt* genes act in these archetypical animals during gastrulation, pattern formation, and regeneration, providing a basic tool set to understand the twist of canonical and noncanonical functions in Wnt signaling.

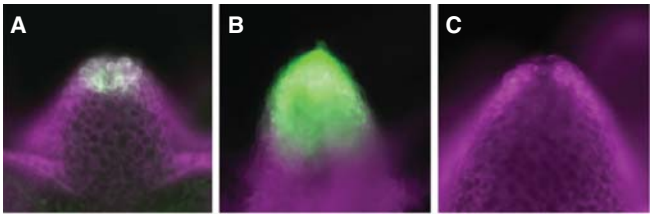

Figure 5. Expression of Wnt3 in the *Hydra* organizer. Reporter constructs for HyWnt3-EGFP (green) were used with an independent transformation marker (HyActin–RFP reporter gene, magenta) to ensure that all cells carry the EGFP reporter gene under examination. (*A*) The transgenic *Hydra* strain shows the localization of Wnt3 in epithelial cells at the tip of the hypostome with a complete Wnt3 promoter (HyWnt3FL). (*B*) Transgenic *Hydra* with a reporter construct lacking the HyWnt3 repressor element show dramatic expansion of expression. (*C*) Transgenic *Hydra* with a reporter construct lacking the HyWnt3 activator sequence including TCF binding sites show no expression. (From Nakamura et al. 2011; reprinted, with permission, from the author.)

Future work should focus on unraveling the *cis*-regulatory control mechanisms (Nakamura et al. 2011) that link the coordinated action of *Wnt* genes (van Amerongen and Nusse 2009).

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