Wnt signaling promotes oral but suppresses aboral structures in *Hydractinia* metamorphosis and regeneration

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SUMMARY

We studied the role of Wnt signaling in axis formation during metamorphosis and regeneration in the cnidarian *Hydractinia*. Activation of Wnt downstream events during metamorphosis resulted in a complete oralization of the animals and repression of aboral structures (i.e. stolons). The expression of Wnt3, Tcf and Brachyury was upregulated and became ubiquitous. Rescue experiments using Tcf RNAi resulted in normal metamorphosis and quantitatively normal Wnt3 and Brachyury expression. Isolated, decapitated polyps regenerated only heads but no stolons. Activation of Wnt downstream targets in regenerating animals resulted in oralization of the polyps. Knocking down Tcf or Wnt3 by RNAi inhibited head regeneration and resulted in complex phenotypes that included ectopic aboral structures. Multiple heads then grew when the RNAi effect had dissipated. Our results provide functional evidence that Wnt promotes head formation but represses the formation of stolons, whereas downregulation of Wnt promotes stolons and represses head formation.

KEY WORDS: Wnt, Tcf, β-catenin, Axis formation, Posterior patterning, *Hydractinia*, Cnidaria, RNAi, Metamorphosis, Regeneration, Organizer, Invertebrate, Oocytes, Gonads, Sperm maturation, Sperm development, Oogonia

INTRODUCTION

In cnidarians, components of the canonical Wnt signaling pathway are thought to be involved in the establishment of the anterior-posterior (AP) axis (Amiel and Houliston, 2009; Broun et al., 2005; Hobmayer et al., 2000; Lee et al., 2007; Müller et al., 2004b; Plickert et al., 2006). The above studies, and others, have shown that several Wnt genes are expressed asymmetrically in the adult form, the polyp, with the highest expression levels around the mouth area (i.e. the oral pole). Other Wnt genes are expressed uniformly in the polyp. It has also been demonstrated that, in *Hydractinia* and *Clytia* embryos, Wnt3, Tcf and *frizzled* are maternally deposited at the pole of the oocyte that corresponds to the future posterior pole of the larvae and the future head, or oral pole, of the adult polyp (Momose and Houliston, 2007; Plickert et al., 2006). Nuclear β-catenin was reported in the corresponding pole of the *Nematostella* embryo (Wikramanayake et al., 2003). Finally, it has been shown that misexpression of Wnt3, *frizzled* (Momose et al., 2008; Momose and Houliston, 2007) and β-catenin (Gee et al., 2010), as well as blocking of GSK3β (Hassel et al., 1993; Müller et al., 2004b), affects AP axis formation, suggesting that a Wnt-mediated organizer acts to specify the position of the cnidarian head (Broun et al., 2005). There is no functional data in the literature on the effect of Wnt pathway inhibition on aboral structures (Tanaka and Weidinger, 2008).

*Hydractinia echinata* is a dioecious colonial marine hydroid common in the European North Atlantic. The *Hydractinia* life cycle is depicted in Fig. S1 in the supplementary material. A *Hydractinia* colony is composed of repeating genetically identical polyps. Two types of polyps predominate: feeding polyps (gastrozooids) and sexual polyps (gonozoids), which carry the gonads (see Fig. S1 in the supplementary material). All polyps in a colony are interconnected by a system of gastrovascular tubes called stolons that enable distribution of food and exchange of stem cells among remote parts of the colony (Müller, 1964; Müller et al., 2004a). Polyps are cylinder-shaped and comprise two epithelial layers, epidermis and gastrodermis, which are separated by a basement membrane, the mesoglea. Polyps are polarized, with a mouth surrounded by tentacles at one end (the oral pole, or head) and stolons at the other end (the aboral pole). Colonies grow by elongation of the stolons from which new polyps bud. Sexual reproduction occurs daily by the release of eggs and sperm into the water by female and male colonies, respectively, in a light-induced spawning event. The embryo develops within 3 days into a planula larva that is competent to metamorphose into a primary polyp (Frank et al., 2001). The direction of movement of the larva is referred to as anterior. This anterior larval pole, however, does not correspond with the head of the polyp. After receiving an external metamorphosing signal, the larva attaches to the substrate by its anterior end, which becomes the aboral pole of the polyp (i.e. stolons), whereas the posterior end of the larva transforms into a head. Wnt3 and Tcf mRNAs are maternally deposited in the prospective oral pole of the unfertilized egg and are later zygotically expressed in the same pole of the embryo (Plickert et al., 2006). In the metamorphosis-competent larva, however, the expression of these genes is significantly downregulated. Expression of Wnt3 and Tcf increases in the posterior end of the larva following the induction to metamorphose and remains present through maturation as a spot of Wnt3-expressing cells at the very oral end of the polyp.

In order to extend our knowledge of the role of the Wnt signaling pathway in cnidarian axis formation downstream of β-catenin stabilization, and to examine a possible effect on aboral development, we have performed functional studies on the specific role of the Wnt downstream transcription factor Tcf in...

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two axis formation events in *Hydractinia* development: metamorphosis and head regeneration. We also studied the specific role of Wnt3 in these processes and performed quantitative gene expression analyses to reveal positive-feedback loops among Wnt genes. Our results suggest an additional role for Wnt in cnidian axis formation purely specifying the site of head development.

**MATERIALS AND METHODS**

**Animals**

*Hydractinia echinata* colonies were collected from Galway Bay by SCUBA diving. They were cultured in natural seawater at 18°C under 14:10 light:dark regimes and were fed brine shrimp nauplii (*Artemia salina*) six times a week. Sperm and eggs were collected daily about an hour after the onset of light and embryos were kept in Petri dishes for 3 days until embryos had completed development into metamorphosis-complete planula larvae. Metamorphosis was induced by a 3-hour pulse treatment of 116 mM CsCl in seawater as previously described (Frank et al., 2001; Müller and Buchal, 1973) and the animals were allowed to settle and metamorphose on glass coverslips. For regeneration experiments, polyps were cut near their bases from adult colonies using fine surgical scissors and their heads removed by a transverse cut just below the hypostome.

**Azaknenpaulette treatments**

Stock solutions of azaknenpaulette (Sigma) at 1 mM were prepared in DMSO and added to seawater to reach a final concentration of 1 µM. Treatments were carried out in the dark at 18°C.

**Obtaining gene sequences**

Degenerate β-catenin primers designed against the *armadillo* repeat region of the Hydra β-catenin protein (GenBank accession no. AAC71437), forward 5'-CAYCARGAGGGCCAAAATG-3' and reverse 5'-YTCATCCNCCCICYCTC-3', were used to isolate a 501 bp fragment from *Hydractinia* cDNA. To obtain full-length sequences, SMART RACE protocol (Clontech) was used for 3'/H11032 fragment from *YTGCATICCNCCIGCYTCIA-3* and reverse 5'-CATCCTTCTCTCTTGGACATCG-3', were used to isolate a 301 bp fragment from *H11032* RACE, and the resulting complementary strands of RNA for each gene were mixed and reverse transcribed to cDNA using Omniscript Reverse Transcriptase (Qiagen). Gene sequences

**RNai experiments**

Templates for RNai synthesis were made by PCR. Two templates were made for each dsRNA, one with a T7 promoter site on the forward primer and a second with a T7 site on the reverse primer. Primer pairs for *Tcf* were as follows: T7 forward 5'-GATCATATAACGACTCATTAGAAGGTCGATCCTTGG-3' and reverse 5'-TGCAGCTTACCTATAGGAGTTTGGTACAG-3', T7 forward 5'-GATCATATAACGACTCATTAGAAGGTCGATCCTTGG-3' and reverse 5'-TGCAGCTTACCTATAGGAGTTTGGTACAG-3'.

**RESULTS**

**Gene sequences**

The full coding sequence of the *Hydractinia* β-catenin showed 84% similarity to the closely related hydroid *Podocoryne carnea* β-catenin and 74% to *Hydra vulgaris* and *H. magnipapillata* β-
catenin at the nucleotide level. At the protein level, it showed 94% and 77% similarity to Podocoryne and Hydra β-catenin, respectively (see Fig. S2 in the supplementary material).

The full coding region of Tcf showed closest similarity to the Hydra magnipapillata Tcf with 67% identity and 76% positives at the protein level (see Fig. S2 in the supplementary material). All new gene sequences have been deposited in GenBank under accession nos. GU145277 for β-catenin and GU145278 for Tcf.

The Hydractinia trans-spliced leader was used to obtain the 5′ sequences of β-catenin and Tcf. This trans-spliced leader is added to many, although not all, Hydractinia transcripts (our unpublished results), which is similar to the situation in Hydra (Stover and Steele, 2001), and can be used to easily obtain full 5′ ends of Hydractinia mRNAs.

Expression pattern of Wnt3, β-catenin and Tcf in normal Hydractinia polyps

As previously reported (Müller et al., 2007), Hydractinia Wnt3 expression was restricted to the prospective oral poles of embryos and larvae (Fig. 1A-C), and to the oral pole of normal polyps in both the epidermis and the gastrodermis (Fig. 1D,E), which is very similar to Wnt3 expression in Hydra (Hobmayer et al., 2000). This pattern was maintained throughout the budding, growth phase and adult life of the polyps, and there was very little variation among different polyps within and between clones. Wnt3 expression also occurred at the apical pole of the gonads as previously described (Fig. 1E) (Müller et al., 2007).

Hydractinia polyps expressed β-catenin ubiquitously throughout their life cycle, with slightly higher levels of expression in the head (Fig. 2I), similar to β-catenin expression in Hydra polyps (Hobmayer et al., 2000). β-catenin was more strongly expressed in the earliest stages of development, until the larval stage (Fig. 2E,F). Its expression was also strongly upregulated in oocytes and during spermatogenesis; however, it was no longer expressed in fully developed mature sperm (Fig. 2G,H).

The peak expression of Tcf in Hydractinia polyps was around the mouth area (Fig. 2D; Fig. 3I). This expression pattern corresponds well with the one observed in Hydractinia embryos (Plickert et al., 2006) and Hydra polyps (Hobmayer et al., 2000). Like β-catenin, Tcf expression was upregulated in oocytes, initially detectable as they migrate from the germinal zone into the developing gonads (Fig. 2A-C). Tcf is known to be a maternal transcript (Plickert et al., 2006). Our results show that the source of maternal Tcf and β-catenin mRNAs is the oocyte itself rather than nurse cells, as might be the case in Hydra (Alexandrova et al., 2005).

Brachyury was expressed in a very similar way to Wnt3 but with a slightly broader expression domain. It was restricted to just a few cells in the oral end of the polyp, confirming previous observations (Fig. 1F; Fig. 3J) (Müller et al., 2007). Throughout development, Brachyury expression was weaker than that of Wnt3 and Tcf. However, in mature feeding polyps, Brachyury mRNA levels are closer to Wnt3 and Tcf as revealed by qPCR (our unpublished results).

Abolition of aboral fate identity during metamorphosis

To determine whether the oral-aboral (OA) axis of the primary polyp is patterned only once, along with the larval AP axis early in development, or is continuously maintained during embryonic development and metamorphosis, Wnt signaling was ectopically activated throughout the metamorphosing larvae. This was done by incubating metamorphosis-induced animals in azakenpaullone, a specific inhibitor of GSK3β (Knick et al., 2004; Teo et al., 2006), thereby mimicking a global Wnt signal.

Larvae were induced to metamorphose for 3 hours (Müller, 1973), then incubated in seawater containing a final concentration of 1 µM azakenpaullone for 18 hours. These treatments resulted in an extreme oralized phenotype in most of the treated animals (70%), with complete absence of aboral structures (stolons) and body columns; however, many ectopic tentacle buds developed from the entire animal (Fig. 3B; see Fig. S3B in the supplementary material). Stolon buds normally develop synchronously with tentacle buds and body columns but the treated animals began budding tentacles without having developed a body column and stolon buds. As metamorphosis continued, these buds went on to form fully developed tentacles. As a result, the animals appeared as balls with tentacles, completely oralized and lacking any non-head features, being virtually ‘floating heads’ (Fig. 3B,P; see Fig. S3C in the supplementary material). The remaining 30% of the animals had a less severe phenotype, with ectopic tentacles on reduced-sized body columns and mostly completely lacking stolons. Quantitative real-time PCR (qPCR) demonstrated that the expression level of Wnt3, itself a target of Wnt signaling, and also the expression of two of its classical target genes, Brachyury and Tcf, were significantly upregulated upon azakenpaullone treatment (Fig. 3Q-S). In situ hybridizations of Wnt3, Brachyury and Tcf mRNA showed that on the day the treatment was ended, azakenpaullone-treated animals expressed all three genes ubiquitously throughout their bodies (Fig. 3M-O). Normally, Wnt3, Brachyury and Tcf expression is restricted to the oral pole of the animal throughout the life cycle, including at this stage (Fig. 3I-K).

During the 24 hours after the azakenpaullone treatments were terminated, Wnt3 expression gradually resumed a normal pattern, becoming restricted to the oral tip as in untreated metamorphosed animals (Fig. 3P). This was followed, with a 3-4 day delay, by the
acquisition of a gradually more normal phenotype, with growth of normal body columns and stolons and loss of ectopic tentacles, showing that AP patterning occurs continuously in *Hydractinia*.

In order to show that the oralized phenotype is the result of ectopic activation of the canonical Wnt signaling, we performed rescue experiments by downregulating the mRNA levels of the Wnt downstream transcription factor Tcf using RNAi. For these treatments, metamorphosing animals were co-incubated in 1 μM azakenpaullone and 80 ng/μl of Tcf dsRNA. These experiments resulted in animals that showed a normal phenotype compared with animals treated with azakenpaullone-only. The rescued polyps had no ectopic tentacles and their body columns and stolons appeared normal (Fig. 3C; see Fig. S3A in the supplementary material). In support of these results, qPCR analysis showed that the net result of the combined azakenpaullone and Tcf RNAi was a normal expression level of Wnt downstream transcription factor Brachyury. Tcf expression remained similar to the RNAi treatment level (Fig. 3Q-S).

To examine the effect of Tcf knockdown alone (i.e. without azakenpaullone treatment), gastrulae 36 hours post-fertilization were incubated for 15 hours in seawater containing Tcf dsRNA. Immediately after this incubation, the now-competent larvae were induced to metamorphose using CsCl for 3 hours. After induction, they were subjected to a second Tcf/RNAi treatment, this time lasting 24 hours. This was done to ensure a prolonged period of Tcf mRNA depletion, given that RNAi downregulated gene expression reappears 30-60 hours after termination of the treatment (Plickert et al., 2003). Tcf knockdown neither affected the ability of the embryos to develop to metamorphosis-competent larvae, nor to begin metamorphosis. The initial stages of metamorphosis, i.e. normal contraction and settlement, also occurred normally up to stages 11-12 (Seipp et al., 2007). However, the treated animals then failed to develop properly patterned primary polyps. By 28 hours post-induction, 30% of the treated animals had failed to develop either stolons or tentacles, compared with 7% of the controls (Fig. 3D,G). A further 39% of treated animals also failed to develop a normal primary polyp by this time. They showed, instead, a general tendency of a shift in the OA balance towards aboral structures (Fig. 3E,F), as described below.

For each animal, total stolon length and the length of the longest tentacle bud were measured at 28 hours after induction of metamorphosis. A head development to stolon development ratio was obtained by dividing an animal’s stolon length by the length of its longest tentacle bud, which was taken as a proxy for the level of head development. This was not done for animals completely lacking stolons or tentacles to avoid division by zero. The data was then coded by assigning each ratio into one of eight groups (Table 1) and the results were graphed to show the shift in the balance between oral and aboral development (Fig. 3H). Group 1 contains animals with no aboral development, but with heads, whereas Group 8 animals had no oral development but had grown stolons. Groups 2-7 were intermediate stages between the two above extremes. The range of stolon:tentacle ratio in control animals fell within Groups 2-6 with the structures of both poles developing synchronously. The data was normally distributed for control animals with 40% in Group 4. Tcf RNAi-treated animals showed completely abnormal development, strongly deviating from normal distribution with only 10% in Group 4. Thirty-nine percent of the treated animals fell into groups unique to the treatment (i.e. no control animals in the group). Only 31% of treated animals

![Fig. 2. Expression patterns of Tcf and β-catenin. (A-D) Tcf. (A) Female polyp. Strong expression is visible in the developing gonads with weaker expression in the polyp head. (B) Young oocytes expressing Tcf as they enter the developing gonad. (C) Tcf expression in two oocytes located in a newly developed gonad. mRNA is concentrated uniformly around the germinal vesicle. (D) Oral (top) view of mature feeding polyp. Tcf expression is visible around the mouth. (E-I) β-catenin. (E) Ubiquitous expression in the early stages of development (16-cell embryo and late gastrula). (F) Expression in the larva is ubiquitous, but increased expression is also seen at the future oral pole in a similar domain as Wnt3 (arrowhead). (G) Female polyp. β-catenin is expressed in the head of the polyp, in early oocytes in the germinal zone and in oocytes in early gonads. (H) Male sexual polyp. β-catenin is expressed in the head of the polyp and during spermatogenesis, but not in mature sperm. i, immature male gonad expressing β-catenin during sperm development; m, mature gonad no longer expressing β-catenin. (I) β-catenin is ubiquitously expressed at low levels throughout mature feeding polyps. Increased expression is seen just under the apical tip of the mouth. h, head (A, G and H). Scale bars: 200 μm in A,D-I; 20 μm in B,C.](image-url)
developed normally, whereas 27% of treated animals showed a lack of only oral development upon Tcf knockdown (Groups 7 and 8) compared with 0% of controls. Unexpectedly, 12% of treated animals developed only heads (Group 1). However, despite not forming stolons, the heads of Group 1 animals, although present, had only developed on average to one quarter the size of the controls (Fig. 3E).

In summary, knocking down of Tcf by RNAi disrupted the balance between oral and aboral development. Oral development was affected in 69% of Tcf RNAi-treated animals, of which 27% were lacking in oral development only, 12% were lacking stolon development but also showing severely reduced oral development, and a final 30% failed to develop oral and aboral structures altogether by 28 hours post-induction. The specificity of the RNAi was demonstrated by the qPCR experiments, which were normalized to GAPDH expression, showing also that the expression of two unrelated genes, 18s rRNA and the stem-cell marker Vasa (Rebscher et al., 2008), remained unchanged (Fig. 3T,U). In addition, normal phenotypes were obtained when Tcf RNAi was done in combination with azakenpaullone (see above).
The role of Wnt in regeneration

To study the role of Wnt in polyp regeneration we used azakenpaullone and RNAi in regenerating polyps. Polyps from mature colonies had their heads removed just beneath the hypostome by a transverse cut. They were then isolated from the colony by cutting close to the stolonal mat without the inclusion of stolonal tissue, resulting in cylinder-like body columns (Fig. 4A).

Normal regeneration in isolated, decapitated Hydractinia polyps proceeded as follows: several hours after cutting, the wounds on either end of the polyp body column had healed. At this stage, no early structures, i.e. tentacle buds, hypostome or stolons, were visible to indicate the OA polarity. A spot of Wnt3 expression, however, became established at one end of the body column alone, indicating that polarity was maintained or re-established within 1 day post-cutting (Fig. 5A). By the next day, i.e. 2 days post-cutting, new head structures became visible, usually as tentacle buds (Fig. 4B). Complete heads could be observed by the third day after cutting at one end of the polyp only, whereas the other end showed no further development after wound healing (Fig. 4F). Rarely,
in<5% of the cases, polyps developed a head at both ends of the body column. Stolon regeneration occurred in <1% of the cases.

To interfere with normal Wnt signaling during regeneration, we used RNAi to downregulate Wnt3 and Tcf mRNA levels in isolated, decapitated polyps. The knockdown was confirmed by in situ hybridization and qPCR. As mentioned above, 1 day after cutting, a spot of Wnt3 expression developed at the future site of head regeneration in decapitated, but otherwise untreated, isolated polyps (Fig. 5A). In polyps that were also treated with Wnt3 dsRNA, however, this spot of expression was absent (Fig. 5F). These results were confirmed quantitatively by qPCR that not only Wnt3, but also two of its target genes, Tcf and Brachyury, were downregulated following incubation in Wnt3 dsRNA (Fig. 6A-C), whereas the expression of two unrelated genes, 18s rRNA and Vasa, were not downregulated (Fig. 6D,E). We also treated polyps with Tcf dsRNA. These experiments gave similar results to Wnt3 RNAi, i.e. specifically downregulating not only Tcf, but also Wnt3 and Brachyury (Fig. 6A-C), while not reducing the expression of unrelated genes (Fig. 6D,E).

In polyp body columns treated with Wnt3 RNAi, head regeneration was inhibited. The earliest head structures (tentacle buds) became visible only 3 or 4 days after cutting (2 or 3 days after the end of treatment) when the effect of the RNAi had dissipated, compared with 2 days after cutting for controls. Interestingly, in contrast to normal regeneration where stolons did not develop, in RNAi-treated animals, stolon buds appeared in 30% of the cases from 2 or 3 days after cutting (1 and 2 days after the end of treatment). Stolons, which were identified by their ability to attach to the substratum (see Fig. S3G-I in the supplementary material), only developed in animals in which no head regeneration was present (Fig. 4K). Another common phenotype only seen in Wnt3 RNAi-treated polyps (15%) was the formation of two abnormally small heads side by side, on the same pole of the polyp, a phenomenon which was never seen in controls but often observed in cut polyps treated with LiCl (Fig. 4M; see Fig. S3E,F in the supplementary material). Five days after cutting (4 days after the end of the treatment), 20% of the treated animals had developed small ectopic polyps protruding from the original body column (Fig. 4M-O). By 13 days after the end of treatment, 15% of RNAi-treated body columns had developed into non-headed, multi-branched stolon-like structures, or small offshoot polyps coming from an intertwined mass of tissue. Rarely (5%) did such tissue knots bud small offshoot polyps at either end (Fig. 4L,N). Downregulation of Tcf resulted in similar phenotypes (Fig. 4G-J). The specificity of Wnt3 and Tcf RNAi treatments was further demonstrated by incubating decapitated polyps in dsRNA corresponding to the backbone of pGEM-T vector. Such treatments resulted in single head formation occurring with the same timing as in polyps allowed to regenerate in the absence of dsRNA (Fig. 4B,C). None of the phenotypes resulting from Wnt3 or Tcf RNAi treatments occurred in the pGEM-T control RNAi treatments. The Wnt3 and Tcf RNAi prevented the re-establishment of a Wnt3 expressing spot, thereby delaying head regeneration. During the absence of normal Wnt3 expression, some polyps formed stolon buds. Once the RNAi had dissipated, multiple Wnt3 expression sites were established in many polyps (Fig. 5). In polyps that went on to develop multiple heads, each head contained a Wnt3-expressing spot (Fig. 5L). Conversely, stolon buds showed no Wnt3 expression (Fig. 5H,M,O).

To study the effect of global Wnt activation on regeneration, we treated isolated decapitated polyps with azaktenaunolone. A 24-hour treatment resulted in the polyps developing two heads, one of them
showed that the mRNA levels of heads alone without any other structures. qPCR experiments head, resulting in completely oralized phenotypes that comprised the polarity of the entire metamorphosing animals towards the Ectopic activation of the Wnt pathway by blocking GSK3 had heads (single or double, respectively) that were fully and 10% of the azakenpaullone-only treated polyps, both of which polyps failed to regenerate heads, in contrast to none of the control ended), 70% of the joint azakenpaullone and complete rescue. Four days after cutting (3 days after treatment stock was not removed and RNAi experiments there showed Ectopic activation of Tcf protein (decapitation), which prevented (or only characteristic of the azakenpaullone phenotype. However, such the effects of the azakenpaullone treatment during metamorphosis, i.e. fully oralized animals.

When azakenpaullone treatments were performed in conjunction with Tcf RNAi, the cut polyps failed to develop the double heads characteristic of the azakenpaullone phenotype. However, such treatments produced animals more similar to polyps treated with only Tcf RNAi than to controls. This might be due to physical removal of the Tcf protein (decapitation), which prevented (or reduced) the azakenpaullone-mediated Wnt downstream events activation. By contrast, during metamorphosis, the Tcf protein stock was not removed and RNAi experiments there showed complete rescue. Four days after cutting (3 days after treatment ended), 70% of the joint azakenpaullone and Tcf RNAi-treated cut polyps failed to regenerate heads, in contrast to none of the control and 10% of the azakenpaullone-only treated polyps, both of which had heads (single or double, respectively) that were fully regenerated by this time.

**DISCUSSION**

Ectopic activation of the Wnt pathway by blocking GSK3β shifted the polarity of the entire metamorphosing animals towards the head, resulting in completely oralized phenotypes that comprised heads alone without any other structures. qPCR experiments showed that the mRNA levels of Wnt3, Tcf and Brachyury were upregulated significantly (and downregulated in the corresponding RNAi experiments). The spatial expression of Wnt3, Brachyury and Tcf became ubiquitous (Fig. 3M-O) in contrast to a polarized expression at the oral ends of untreated polyps (Fig. 3I-K). As global Wnt activation completely abolished the development of aboral structures, the azakenpaullone-treated animals appeared ‘delayed’ because they remained ball-like as normal animals appear during early metamorphosis. Tentacle budding, however, occurred simultaneously with the control animals, showing that no genuine delay resulted from the azakenpaullone treatment.

Rescue experiments of the azakenpaullone-treated animals by downregulating Tcf with RNAi provided direct evidence for the function of Tcf and, therefore, the entire canonical Wnt pathway in axial patterning. The RNAi rescue experiments restored normal metamorphosis in terms of timescale, morphology and quantitative Wnt3 and Brachyury expression (Fig. 3), showing that deregulation of the canonical Wnt signaling alone is sufficient to generate aberrant phenotypes not only in the oral pole, as previously thought, but also in the aboral pole.

In metamorphosing animals treated only with RNAi for Tcf, there was a general shift in roughly half of the affected animals towards aboral (i.e. body columns and stolons) development at the expense of head structures, as would be expected (Broun et al., 2005; Lengfeld et al., 2009). In the second half of affected animals, development of both poles was inhibited. In these animals, heads were one quarter the size of controls, indicating that head formation in this group was also affected by the treatment.

Wnt3 was expressed in the oral tip of normal polyps. However, if the head was removed, a new Wnt3-expressing tip formed within 24 hours (Fig. 5A), followed by regeneration of new head structures about 2 days later. Wnt3 RNAi downregulated the expression of Wnt3 and prevented head regeneration until the RNAi effect dissipated (shown by both in situ hybridization and qPCR). Downregulation of Wnt3 in regenerating Hydra also prevented regeneration, but the treated animals died within 3 days (Chera et al., 2009). qPCR on Tcf and Brachyury, both classical Wnt target genes in other animals (Hovanes et al., 2001; Yamaguchi et al., 1999), showed that their expression also went...
down and, thus, that these genes are also Wnt3 targets in Hydractinia. Complementary experiments in which Tcf mRNA levels were downregulated by RNAi showed matching results, i.e. downregulation of Wnt3 and Brachuryv in addition to Tcf itself.

Downregulation of Wnt3 and Tcf by RNAi prevented head regeneration. This result was expected if Wnt signaling was essential for head formation. The inhibition of the canonical Wnt signaling by either Wnt3 or Tcf RNAi, however, had no effect on wound healing, which continued without forming a head. Many body columns elongated and were transformed to stolon-like tissue. Heads only developed after the effect of dsRNA weakened, about 2-3 days after the end of treatment. Surprisingly, extra head structures appeared ectopically. This might be seen as a paradox because ectopic heads also appeared following global activation of Wnt. Our interpretation for this phenomenon is that the absence of a dominant head organizer, known from experiments in Hydra to emit head-inhibiting signals (MacWilliams, 1983), prevented the production of such inhibitory signals. Numerous head organizing spots were thereby able to establish themselves once the effect of RNAi was weak enough. Consistent with this view, multiple Wnt3-expressing spots were observed in RNAi-treated polyps and each ectopic head expressed the gene (Fig. 5). These results indicate that head inhibitory signals are generated downstream of Wnt. The overall effect was similar in both Wnt3 and Tcf downregulation, indicating complete functional conservation of the canonical Wnt pathway in Hydractinia. Our data show that the polarity of the polyp is only maintained in the presence of a Wnt signal at one pole. After the removal of the Wnt3-expressing head, the polarity is maintained by a newly established Wnt3-expressing organizer within 24 hours. If, however, Wnt signaling is downregulated (by RNAi) for a longer period, the polyp will completely lose its polarity and the tissue is capable of adopting any positional value in a stochastic fashion, and this explains the various phenotypes resulting from both Wnt3 and Tcf RNAi (Fig. 4).

Stolon regeneration did not occur in normal polyps, confirming earlier findings that stolon regeneration occurs only in young polyps of Hydractinia (Müller et al., 1986) or when animals are incubated in the uncharacterized stolon-inducing factor (Müller et al., 2004b). However, stolon buds appeared in RNAi-treated regenerating animals (both Wnt3 and Tcf), whereas head regeneration was inhibited. Furthermore, the ‘floating head’ phenotype, from azakenpaullone treatment, demonstrated that Wnt signaling was sufficient to prevent stolon formation. We propose that colonial hydroid heads emit, in addition to the head-inhibiting signal mentioned above, a stolon-inhibiting signal. This would allow stolon development only at the aboral end, away from the inhibitor source (i.e. the head), where the signal concentration is low enough. This is consistent with Meinhardt’s model on the long-range inhibitory effect of the Wnt-expressing pole (Meinhardt, 2009). Disrupting Wnt signaling by RNAi might thus remove the stolon inhibitory source and allow ectopic stolon development.

Wnt signaling is involved in axial patterning throughout the Metazoa. It controls formation of posterior structures in most bilaterians (Niehrs, 2010; Petersen and Reddien, 2009). In planarians, Wnt directs regenerating tissue to a posterior fate, whereas inhibition of Wnt directs regenerating tissue to develop anterior structures (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Although such Wnt-mediated control of posterior formation initially appears to be the opposite of its oralizing role in cnidarians, this is not the case. The cnidian oral pole is thought to be equivalent to the bilaterian posterior pole (Meinhardt, 2002). Indeed, Fig. 1C clearly shows that the Wnt3-mediated organizer is located at the posterior tip of Hydractinia larvae, the future location of the oral pole.

Taken together, our results show functionally that: (1) Tcf-dependent Wnt signaling is essential and sufficient to induce oral (i.e. posterior) patterning and the subsequent development of oral structures; and (2) that the axial patterning of the entire animal is mediated by Wnt, through a graded stolon (i.e. anterior) inhibitory effect of the head organizer. This study reinforces the results obtained by others, indicating that Wnt-mediated axis formation is a basal character in eumetazoa.

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Competing interests statement
The authors declare no competing financial interests.

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References


PodBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPGSTMADILTTQSYYQDELYSPHVTTQSQ 776
HeBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPNTEMADILTTQSYYQDELYSPHITQSQ 776

PodBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPGSTMADILTTQSYYQDELYSPHVTTQSQ 776
HeBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPNTEMADILTTQSYYQDELYSPHITQSQ 776

PodBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPGSTMADILTTQSYYQDELYSPHVTTQSQ 776
HeBcat: RMSEDKSQDYKKRLSVELTSSLFRDDMPWEPNTEMADILTTQSYYQDELYSPHITQSQ 776