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cWnt signaling modulation results in a change of the colony architecture in a hydrozoan

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ABSTRACT

At the polyp stage, most hydrozoan cnidarians form highly elaborate colonies with a variety of branching patterns, which makes them excellent models for studying the evolutionary mechanisms of body plan diversification. At the same time, molecular mechanisms underlying the robust patterning of the architecturally complex hydrozoan colonies remain unexplored. Using non-model hydrozoan *Dynamena pumila* we showed that the key components of the Wnt/β-catenin (cWnt) pathway (β-catenin, TCF) and the cWnt-responsive gene, *brachyury 2*, are involved in specification and patterning of the developing colony shoots. Strikingly, pharmacological modulation of the cWnt pathway leads to radical modification of the monopodially branching colony of *Dynamena* which acquire branching patterns typical for colonies of other hydrozoan species. Our results suggest that modulation of the cWnt signaling is the driving force promoting the evolution of the vast variety of the body plans in hydrozoan colonies and offer an intriguing possibility that the involvement of the cWnt pathway in the regulation of branching morphogenesis might represent an ancestral feature predating the cnidarian-bilaterian split.

1. Introduction

Cnidarians occupy a sister phylogenetic position to bilaterians, which makes them very valuable for evolutionary developmental biology (Kayal et al., 2018). The remarkable feature of colonial cnidarians is their continuous growth and morphogenesis throughout the whole life of the colony, which makes them convenient models for investigating the mechanisms of the establishment and patterning of the body plan.

Colonial hydrozoans display a wide range of morphologies reflecting their diverse life histories, habitats, and developmental programs (Cartwright, 2004). Colonies of some hydrozoan cnidarians consist of stolons spreading over the substrate, and the hydranths (feeding zooids) budding from the stolons. This type of colony is called the stolonal colony (Fig. 1a1). But thecate hydrozoans (i.e. hydrozoan species from the order Leptothecata which are covered with a chitinous exoskeleton) form colonies of high complexity and architectural sophistication. Despite the fact that each individual zooid of mostly all thecate hydrozoans has a very simple structure and radial symmetry, entire colonies demonstrate various levels of complexity and types of symmetry. Their complex colonies form upright shoots, made of repetitive internodes on which zooids are located. Colonies grow due to morphogenetic activity of the specific organs – the growth tips. The shoot growth tip of thecate hydroids undergoes cycles of morphogenetic processes resulting in the formation of new internodes and nodes, which generate the architecture of the colony (Fig. 1a2, 1a3) (Beloussov et al., 1972; Kosevich and Fedosov, 2008).

The majority of cnidarian Evo-Devo studies are focused on the body patterning of non-colonial animals, such as *Nematostella* and *Hydra*, or on the athecate colonial hydrozoan *Hydractinia*, which forms stolonal colonies (Genikhovich and Technau, 2017; Hobmayer et al., 2000; Sanders et al., 2014). At the same time, the molecular mechanisms underlying the

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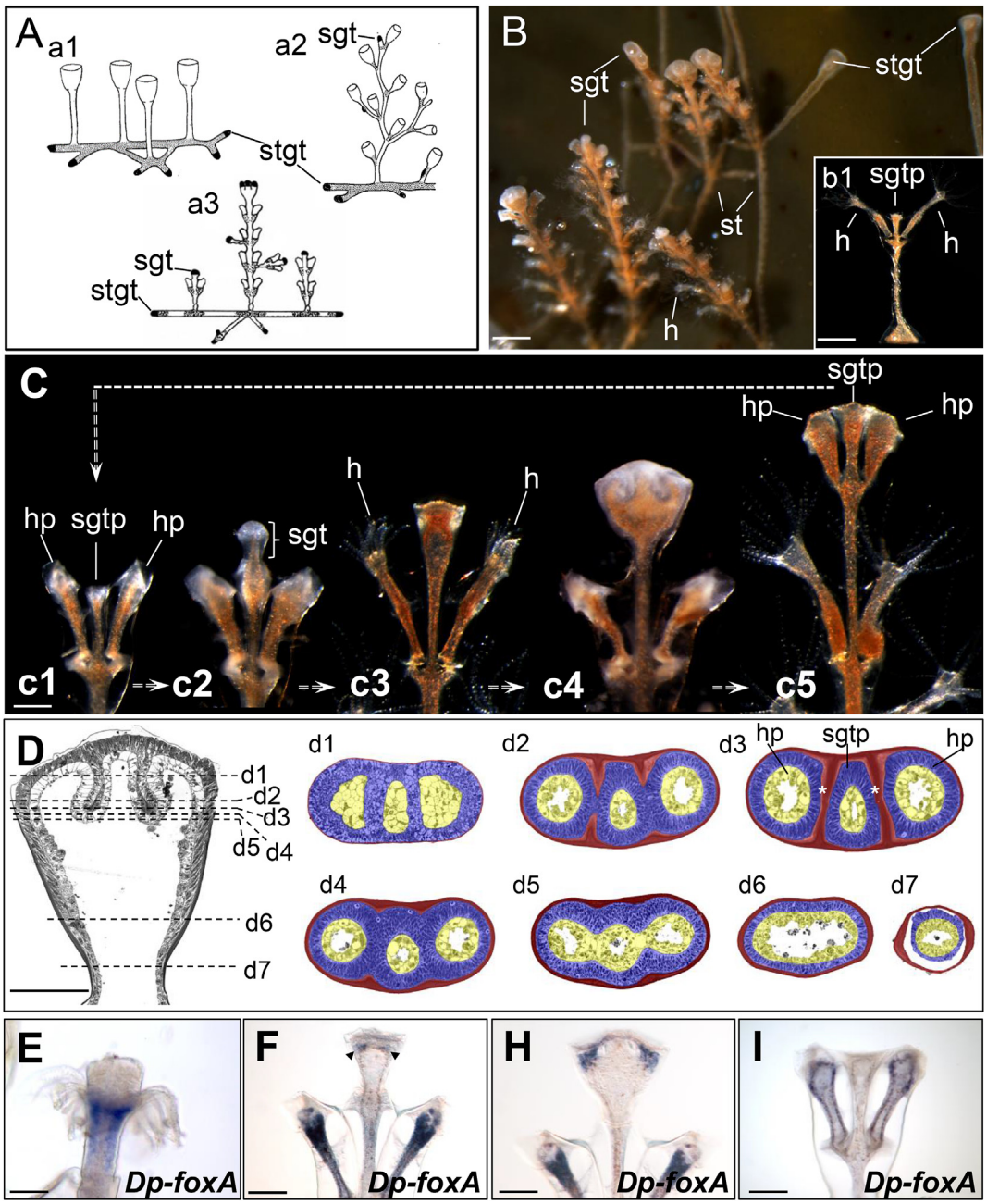

Fig. 1. Morphogenesis and differentiation of the shoot growth tip in *Dynamena* colony. (A) Colony types in thecate hydrozoans (modified after Kuhn, 1914 (Kuhn, 1914)): (a1) stolonal colony; (a2 - a3) colonies shoots with numerous hydranths: (a2) colony with sympodial growth; (a3) colony with monopodial growth and terminal shoot growth tips. (B) Colony of *Dynamena pumila*; (b1) primary shoot of *Dynamena* colony. (C) Successive stages of *Dynamena* shoot growth tip morphogenesis. Arrows indicates succession of developmental stages. (D) Longitudinal section of shoot growth tip. (d1-d7) A series of transversal sections of shoot growth tip, red – perisarc, blue – ectoderm, yellow – endoderm, asterisks mark the chitinous septae. (E–I) Expression patterns of *Dp-foxA* in the hydranth and in the shoot growth tip during morphogenesis. Arrowheads point to oral area endoderm of the forming hydranths. sgtp, shoot growth tip primordium; hp, hydranth primordium; h, hydranths; sgt, shoot growth tip; stgt, stolon growth tip; st, stolon. Scale bar: (B) 300 μM, (b1) 200 μM, (C–I) 100 μM.

patterning of the architecturally complex thecate colonies, remains unexplored. Which molecular mechanisms regulate the robust formation of the complex colony structure? A bulk of evidence supports the idea that diversification of body plans have arisen at least in part by co-option of existing conservative developmental mechanisms (Lanna, 2015; Sanetra et al., 2005). In our work we focused on canonical Wnt (cWnt) signaling pathway which participates in specification and patterning of body axis in animals from sponges to mammals (Petersen and Reddien, 2009). We have shown the involvement of cWnt signaling in spatial patterning of the architecturally complex hydrozoan colony of *Dynamena pumila*.

2. Material and methods

2.1. Animal collection

Dynamena pumila colonies were collected at the Pertzov White Sea Biological Station of the Lomonosov Moscow State University (66°34′ N, 33°08′ E) in June–July. Colonies with mature eggs were collected during low tide and kept in the laboratory at +14 –+16°C in natural sea water (NSW). Extracted embryos and planulae were kept in Petri dishes at +18°C in NSW filtered through Millipore glass filters (0.24 μm) (filtered sea water, FSW). Planulae were stimulated to metamorphosis by

treatment with 100 mM CsCl (Seipp et al., 2006). Whole-mount observations were made under a stereomicroscope Leica M165C.

2.2. Histology

For light microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodilic acid buffer (pH 7.2) and then post-fixed in 1% osmium tetroxide in the same buffer. Samples were then dehydrated through a graded series of ethanol and acetone, and then infiltrated and embedded in Epon812 resin. Samples were sectioned using a diamond knife on a Reichert Ultracut E ultramicrotome. Semi-thin sections were stained by 1% toluidine blue in 1% Na-tetraborate and examined with a Carl Zeiss microscope Axiovert 25 CFL with a Zeiss AxioCam HRc digital camera.

2.3. Chemical treatments

For pharmacological treatments, colony shoots were exposed to chemicals from the beginning of new internode formation. Planulae were incubated in chemicals from the beginning of metamorphosis. 1-Azakenpaullone (Sigma, A3734, manufactured in Canada or China) was used to activate Wnt/βcatenin signaling. Tcf/β-catenin inhibitor iCRT14 was used to inhibit Wnt/β-catenin signaling (Sigma, SML0203, manufactured in USA or China). All reagents were dissolved in DMSO at higher concentrations, and then diluted in FSW to the final concentrations. Treatments were performed with concentrations specified in the main text. Working solutions with the chemicals were refreshed daily.

2.4. Whole mount in situ hybridization

Whole mount *in situ* hybridization of *Dynamena* shoots and primary polyps was performed as described previously (Genikhovich and Technau, 2009). Briefly, samples were fixed for 1 min in 0.2% glutaraldehyde/3.7% formaldehyde in FSW and then for an additional hour in 3.7% formaldehyde/FSW. After 3 PTw (1x PBS with 0.1% Tween 20) washes, the samples were transferred into methanol and stored no more than overnight at −20 °C. Samples were hydrated with PTw and Proteinase K treatment was carried out at 80 μg/ml for 3 min. Samples were hybridized at 62 °C with digoxigenin-labelled antisense RNA probes (1 ng/μl), which were transcribed from linearized plasmids containing *Dynamena* genes. After treatment with alkaline phosphatase-conjugated anti-DIG antibody (Roche; ref 11093274910, 1/2,000 diluted), a chromogenic reaction was performed with BCIP/NBT (Roche). For some genes (e.g. Wnt3) to increase the signal we digested the chitinous perisarc of the colony with chitinase (1 mg/ml) during 30 min at room temperature. A urea-based i*n situ* hybridization method was used for the hydranths (Sinigaglia et al., 2017). Stained samples were washed with PTw, mounted in 87% glycerol and observed under a microscope Leica DM2500.

2.5. Isolation of Dynamena genes

cDNA fragments of *Dynamena* genes for *in situ* hybridization were isolated by PCR from an embryonic cDNA library using specific primers (see Table S1). PCR products were cloned into the pAL-TA vector (Evrogen, Russia). Orthologous relationships between *Dynamena* genes and previously reported genes were confirmed via best BLAST hits. Gene sequences were obtained from the sequenced transcriptome (Illumina HiSeq 4000) of *Dynamena*.

2.6. Reverse transcription, PCR and quantitative real-time PCR

1 mg of total RNA was used for cDNA synthesis by M-MLV reverse transcriptase (Evrogen, Russia) and random hexanucleotides (Evrogen, Russia). PCR was performed on an MJ Mini thermal cycler (BioRad, USA) using Encyclo polymerase (Evrogen, Russia) and specific oligonucleotides. The PCR products were analysed by 1% agarose gel electrophoresis with ethidium bromide (0.5 mg/ml). Quantitative PCR was performed using a Step One plus (Applied Biosystems) and Syber-Green/ROX reaction mixture (Evrogen, Russia). The relative expression levels were calculated using the efficiency-corrected ΔΔCt method (Bookout et al., 2005). For primers for qPCR, see Table S1.

2.7. Statistical analysis

All experiments were conducted at least three times. Total number of samples in each experimental point was no less than 100. All statistical analyses were performed with a one-sided, unpaired Student's t-test. The data are shown as the mean ± s.e.m. $P < 0.05$ was considered to indicate statistical significance. For gene relative expression levels graphs was used ±s.d. as error bars. Statistical analysis was conducted in software package Statistica 6.0 or GraphPad PRISM 5.00.

2.8. Image processing

Microscopy images were processed with Photoshop CC program. The following tools were used for processing: "Levels" for RGB channel, "Brightness", "Contrast" and "Exposure". All tools were applied to the entire image, not locally.

2.9. Data availability

Nucleotide sequences of *Dynamena* genes used in this study have been deposited in the GenBank: Dp-bra1 (MK005873), Dp-bra2 (MK005874), Dp-wnt3 (MK005877), Dp-βcat (MK005875), Dp-tcf (MK005876), Dp-foxA (MK005878), Dp-GAPDH (MK005879).

Transcriptome Shotgun Assembly project used in this study has been deposited at DDBJ/ENA/GenBank under the accession GHMC00000000. The version described in this paper is the first version, GHMC01000000.

3. Results and discussion

3.1. Dynamena shoot growth tip becomes prepatterned before the physical separation of a hydranth's primordia

Our model species, the thecate hydrozoan *Dynamena pumila*, forms architecturally complex colonies possessing biradial symmetry (Fig. 1a3,B). *Dynamena* colony is considered as monopodially growing one. The shoots of this colony consist of repetitive structural units or modules, which we call 'internodes': two lateral hydrants and a fragment of the shoot in between them. The main shoot grows continuously upward repeating the same morphogenetic cycle multiple times. The terminal module is the youngest one, and it consists of three yet undifferentiated parts: primordia of the two lateral zooids and the central part (Fig. 1c1,c2). In the beginning of a new internode formation, shoot growth tip has a hemispherical shape (Fig. 1c2). Then it divides into three parts: the central part produces new fragment of the shoot with the new terminal growth tip, while the lateral parts differentiate into hydranths (Fig. 1c3 - c5). The splitting of the shoot growth tip starts from the formation of two pairs of chitinous septae growing centripetally toward each other from the exoskeleton. Eventually, the opposing septae fuse, dividing the shoot growth tip into three parts (Fig. 1D-d1 to 1D-D6).

To find out when the identities of these parts become determined, we examined the expression of the forkhead box gene *foxA* (Fig. 1E–I), which is a known oral and foregut marker gene in cnidarians and bilaterians (Fritzenwanker et al., 2004; Kostyuchenko et al., 2018; Kraus et al., 2015; Martindale and Hejnol, 2009). In fully formed differentiated hydranths of *Dynamena*, *Dp-foxA* is strongly expressed in the endoderm behind the mouth opening (Fig. 1E). The same pattern is shown in the *Clytia* hydranth (Kraus et al., 2015). At the early stages of a new shoot growth tip development, two zones of *Dp-foxA* expression appear. *Dp-foxA* expression is localized in the oral areas of future hydranths, but the central part remains free of *Dp-foxA* expression (Fig. 1F–I). Thus, the prepatterning of

the shoot growth tip occurs before its physical separation of the hydranth primordia from the central part producing the new shoot growth tip.

3.2. Spatial expression of cWnt signaling components suggest their role in the shoot growth tip patterning

There have been several attempts to explain the development of the *Dynamena* colony (Banghart et al., 2006; Berking et al., 2002; Marfenin et al., 1995), but, the molecular mechanisms underlying morphogenesis of *Dynamena* shoot growth tip remain obscure. Wnt/β-catenin (cWnt) signaling plays a key role in the specification and patterning of the oral-aboral axis in cnidarians, and the Wnt3 protein initiates this cascade in Hydrozoa (Duffy et al., 2010; Kraus et al., 2016; Leclère et al., 2016; Lengfeld et al., 2009; Marlow et al., 2013; Nakamura et al., 2011; Wlkramanayake et al., 2003). We therefore examined the spatial expression of cWnt signaling components *Dp-wnt3*, *Dp-βcat*, *Dp-tcf* and cWnt-signaling responsive genes *Dp-bra1* and *Dp-bra2* during shoot growth tip morphogenesis to check whether these genes may potentially be involved in the shoot growth tip patterning.

In the hydranth, *Dp-wnt3* is expressed at the tip of the hypostome around the mouth (Fig. 2A and B). In the shoot growth tip at the time when it starts splitting (Fig. 1C4,D), *Dp-wnt3* is expressed uniformly in the entire apex ectoderm, i.e. in both the hydranth rudiments and in the central part (Fig. 2C–F). This pattern was observed through all stages of the shoot growth tip morphogenesis until the completion of the separation of the three parts of the tip by septae. Then *Dp-wnt3* expression vanishes in the central part giving rise to the new shoot growth tip (Fig. 2G).

Next, we examined expression patterns of the genes encoding two transcription cofactors, *Dp-βcat* and *Dp-tcf*, which regulate the expression of the genes downstream of the cWnt pathway. *Dp-βcat* is expressed in the entire shoot growth tip at the beginning of its growth (Fig. 2H). Starting from the septae formation, two distinct lines of strong *Dp-βcat* expression appear corresponding to the future borders between the hydranth primordia and the central part (Fig. 2I,I′). The pattern of *Dp-tcf* expression (Fig. 2K,K') looks very similar to that of *Dp-βcat*. From this, we can conclude that *Dp-βcat* and *Dp-tcf* demarcate the future borders separating the hydranth primordia from the central part.

It was shown that the expression of *Hydra* genes *brachyury1* and *brachyury2* is regulated by cWnt signaling (Bielen et al., 2007), and *brachyury* was suggested to play a key role in maintaining the activity of the cWnt cascade as a component of a positive feedback loop (Meinhardt, 2012). That is why we studied the expression of *Dp-bra1* and *Dp-bra2* during the morphogenesis of the shoot growth tip.

As expected, expression of both genes was detected in the hypostome of the hydranth (Fig. 2L,P). *Dp-bra1* is expressed uniformly in the ectoderm at a low level through shoot growth tip morphogenesis (Fig. 2M–O). The expression pattern of *Dp-bra2* is remarkably different. From the very beginning of the shoot growth tip formation, *Dp-bra2* is expressed in the ectoderm in two narrow domains at the opposite sides of

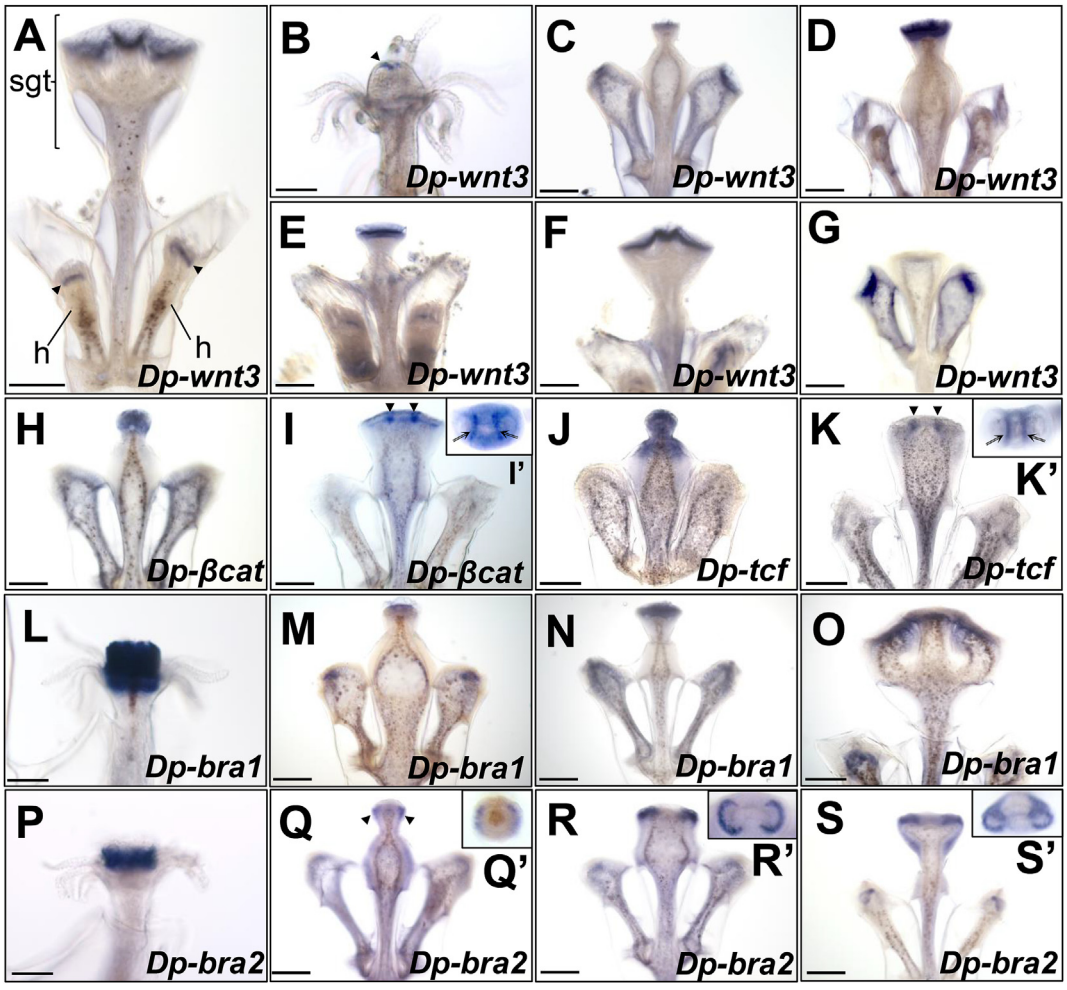

Fig. 2. Spatial expression of cWnt pathway components during shoot growth tip morphogenesis and in the hydranths in *Dynamena* colony. (A–G) *Dp-wnt3*, (H–I) *Dp-βcat*, (J–K) *Dp-tcf*, (L–O) *Dp-bra1*, (P–S) *Dp-bra2*. On (A) and (B) arrowheads point at the *Dp-wnt3* expression in hydranths. In (I–I′) and (K–K′) arrowheads and arrows point at the expression areas of *Dp-βcat* and *Dp-tcf* along the lines of shoot growth tip division. On (Q) arrowheads point at the biradial expression of *Dp-bra2* in the presumptive hydranth areas at the early stage of shoot growth tip morphogenesis. Scale bar: 100 μM.

the shoot growth tip apex (Fig. 2Q,Q′). Then, the expression domains acquire a semicircular shape, and it becomes clear that the expression is localized in the apices of the hydranth primordia (Fig. 2R,R′). The open sides of these semicircles face towards the central part of the shoot growth tip and likely correspond to the stripes of strong *Dp-βcat* and *Dp-tcf* expression. Later, semicircular expression domains close, demarcating the hypostomes of the forming hydranths (Fig. 2S,S'). At this stage, the expression of *Dp-bra2* was also detected in the ectoderm of the whole hydranth primordium (Fig. 2S). It seems that the central part of the dividing shoot growth tip can be distinguished from the lateral parts owing to the absence of expression of *Dp-bra2*.

In summary, we have shown that three genes involved in cWnt signaling and at least two putative cWnt target genes are expressed during the shoot growth tip patterning (Fig. S1). *Dp-wnt3* and *Dp-bra1* are expressed ubiquitously at the top of the developing shoot growth tip. *Dp-βcat* and *Dp-tcf* demarkate the borders of the central part of the shoot growth tip at the lines of its separation from the hydranth primordia. *Dp-bra2* outlines the hydranth primordia. We hypothesize that these three genes (*Dp-βcat, Dp-tcf* and *Dp-bra2*) might be involved in the patterning of the shoot growth tip.

3.3. cWnt signaling is involved in the formation of growth tips, including their specification and morphogenesis

In cnidarians, the establishment and maintenance of the oral-aboral body axis during all stages of development are dependent on cWnt signaling (Lengfeld et al., 2009; Momose et al., 2008; Nakamura et al., 2011). Hyperactivation of the cWnt pathway via chemical inhibition of GSK3β by azakenpaullone (Azk) (Fig. 3A) results in an inhibition of aboral structure development and the disappearance of aboral marker gene expression. Conversely, disturbance of β-catenin and TCF interaction by iCRT14 (Fig. 3A) leads to an expansion of aboral markers and inhibition of oral structures formation (Marlow et al., 2013). To test the role of the cWnt pathway in shoot growth tip patterning in *Dynamena*, we cut off the colony shoots with shoot growth tips at the stage shown on Fig. 1c2 and exposed them to Azk or iCRT14. Upon 36h of 10 μM Azk treatment, shoots formed new internodes lacking the central part, which normally gives rise to the new shoot growth tip (Figs. S2A–D). Application of the 10mkM iCRT14 caused instant arrest of the shoot growth tip (Fig. S2E).

To study a series of morphogenetic cycles of internode formation, we used primary colony shoots (Fig. 1b1) formed from the larvae in the course of metamorphosis under the continuous Azk incubation. Treatment was started right after the larva settlement and stopped after 72 h, when the metamorphosis has been completed. We obtained four clearly distinct phenotypes: 'wild type' phenotype and three affected ones (Fig. 3B–F). The low fraction (ca. 20%, N = 105) of 'wild-type' primary colonies consisting of several internodes were obtained only under the low concentration of Azk (100 nM) (Fig. 3B). Under the higher Azk concentrations (from 1 μM to 10 μM), we obtained three affected phenotypes, all of which lacked the shoot growth tip and formed only the first colony internode (Fig. 3D–F): in the first phenotype, the shoot growth tip divided into three parts, and all of them differentiated into hydranths (Fig. 3D); in the second one, the shoot growth tip divided into two parts, and both became hydranths (Fig. 3E); and in the third one, the shoot growth tip entirely differentiated into a single hydranth (Fig. 3F). We found that phenotypes formed in a dose-dependent manner: the number of single-hydranth primary shoots increased with the increase of Azk concentrations (Fig. 3B). At the Azk 10 μM concentration, more than 75% (N = 106) of primary shoots have only one hydranth.

We showed that at least two cWnt signaling components and one putative cWnt-dependent gene could be involved into the patterning of the shoot growth tip (*Dp-βcat, Dp-tcf, Dp-bra2*). That is why we assayed the expression of these genes in the primary colony terminal internodes upon the continuous 10 μM Azk incubation. Hyperactivation of the cWnt pathway leads to much stronger expression of these genes in the shoot growth tip apex (Fig. 3G). The spatial patterns of expression of all these genes lost all signs of biradial symmetry clearly visible in control shoot growth tips (Fig. 3G). Also, putative cWnt downstream gene, *Dp-foxA*, lose its biradial expression pattern in response to 10 μM Azk treatment (Fig. S3). Moreover, in contrast to controls, all of these genes expressed uniformly throughout the shoot growth tip apices in Azk-treated colonies and did not disappear from their central part (Figs. 3G and S3). Azk treatment changes not only spatial expression pattern but also increase the mRNA expression level of all studied genes (Fig. S2F).

We speculate that the normal course of shoot growth tip morphogenesis is regulated by the differential expression of genes involved in cWnt signaling (at least *Dp-βcat*, *Dp-tcf* and *Dp-bra2*) across the shoot growth tip apex, especially by the differences in their expression in the hydranth primordia vs. the central part. It is likely that alterations in the patterns of gene expression upon increased cWnt signaling activity led to a failure of the central part to acquire its normal identity and to the inability of the shoot growth tip to divide into three parts.

3.4. Long-term hyperactivation of cWnt signaling changes the colony type in Dynamena

The absence of the shoot growth tips is a characteristic feature of a stolonal colony (Fig. 1a1). Surprisingly, we have observed a similar phenotype, i.e. the abolishment of the shoot growth tips, upon cWnt signaling hyperactivation in *Dynamena*. Therefore, we asked whether it is possible to generate a functional colony morphologically distinct from the wild-type colony by changing the activity of only one signaling pathway, namely the cWnt. To test this, we incubated primary colonies in 5 μM Azk for 21 days from the beginning of larval metamorphosis on. Hyperactivation of the cWnt signaling affects not only the development of shoot, but also the formation of stolons. The primary colonies under long-term Azk treatment did not form shoot growth tips within 21 days of the experiment, but produced several stolon growth tips earlier and at a higher percentage than the control ones in a concentration-dependent manner (Fig. 3H). The growing stolons produced the buds of secondary shoots, which formed mostly one hydranth instead of an upright shoot consisting of several internodes, as in the control group (Fig. 4A–C).

Long-term (21 days) down-regulation of cWnt signaling by iCRT14 at the concentration of 5 μM and higher arrested both the shoot growth tip functioning, and formation of the growth tip of stolons (Fig. 4D and E). This confirms that in *Dynamena*, the activity of the cWnt pathway is obligatory for initiation and differentiation of both types of growing tips: the shoot and the stolon growth tips.

In summary, we have shown that changes in the activity of the cWnt pathway is sufficient for the radical modification of the spatial organization of a colony. In the experiment, we observed the replacement of the monopodially growing colony by a stolonal one, which is characteristic for other hydrozoan species, for example for the well-known models athecate *Hydractinia* and thecate *Clytia hemisphaerica* (Fig. 4F and G).

3.5. Washout experiments confirm the role of cWnt activity in the establishment and evolutionary modifications of hydrozoan body plan

To confirm the role of cWnt signaling in the regulation of colony architecture, we conducted washout experiments on Azk- or iCRT14-treated primary colonies. Primary colonies were incubated for 7 days in Azk- or iCRT14 from the beginning of larval metamorphosis. Following washout at the 7th day primary colonies were cultivated further on. In the colonies that underwent incubation in Azk at a concentration of 1 μM and higher, we observed the recovery of wild-type upright branching. Wild-type internodes developing functional shoot growth tips formed from the buds, which appeared on the stolons after the washout of Azk (Fig. S4A). In the experiments with lower Azk concentration (100 nM) recovery of shoot growth tip occurred in the affected primary internodes, even with single hydranth (Figs. S4B and S4C). In some cases, the shoots developed with branching pattern characteristic

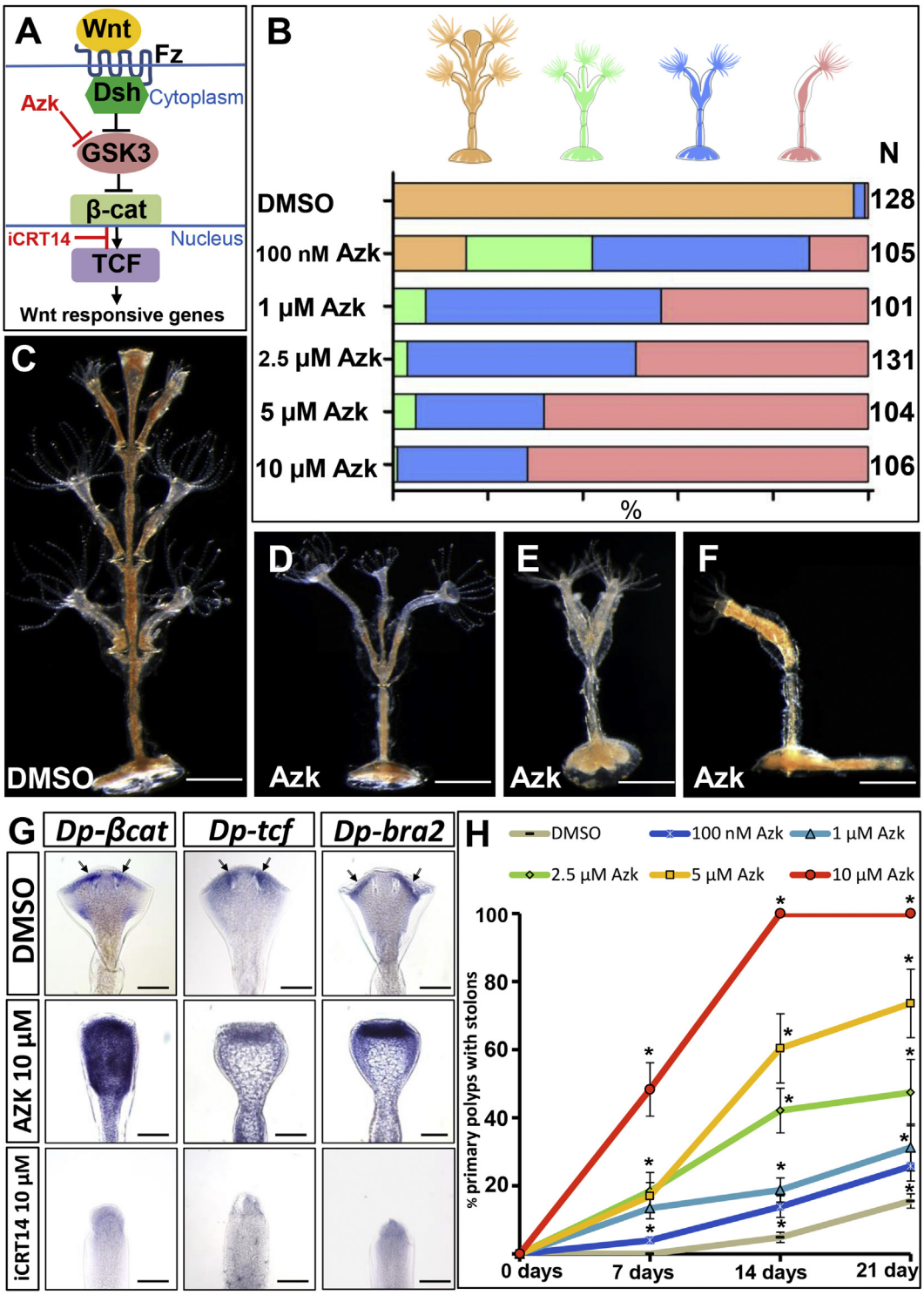

Fig. 3. Activation of cWnt signaling changes the body plan of the *Dynamena* primary colony. (A) Scheme of upregulation and downregulation of cWnt signaling by Azk and iCRT14 treatments respectively. (B) Metamorphosing larvae treated by Azk developed into radialized one-hydranth primary polyp in dose dependent manner. (C–F) Azk treatment results in the inhibition of a new shoot growth tip specification in the primary module: (C) DMSO, (D–F) Azk phenotypes. (G). Expression patterns of *Dp-βcat*, *Dp-tcf* and *Dp-bra2* in response to cWnt activity modulation. Overactivated cWnt signaling leads to upregulated and radialized expression of *Dp-βcat*, *Dp-tcf* and *Dp-bra2*. Arrows indicate biradial patterns of gene expression (H) Dose-dependent activation of stolon growth tip formation by Azk treatment. The data are presented as mean ± s.e.m. *, $P < 0.05$ compared with control (DMSO); one-sided *t*-test. Scale bar: (C–F) 300 μM, (G) 100 μM.

not for *Dynamena*, but for other Sertulariidae species, for example, *Sertularella gigantea* (Figs. S4C and S4D). In *Sertularella*, shoot growth tip divides into two parts: the shoot growth tip primordium and one hydranth (Fig. S4D).

These results suggest that a fine-tuning of the cWnt signaling activity might be responsible not only for the switch between the stolonal and branching types of colony, but also for the changes in the spatial organization of the branching colonies.

As shown here, inhibition of cWnt activity by iCRT14 arrests the development of the shoot growth tip in the primary colony as well as the

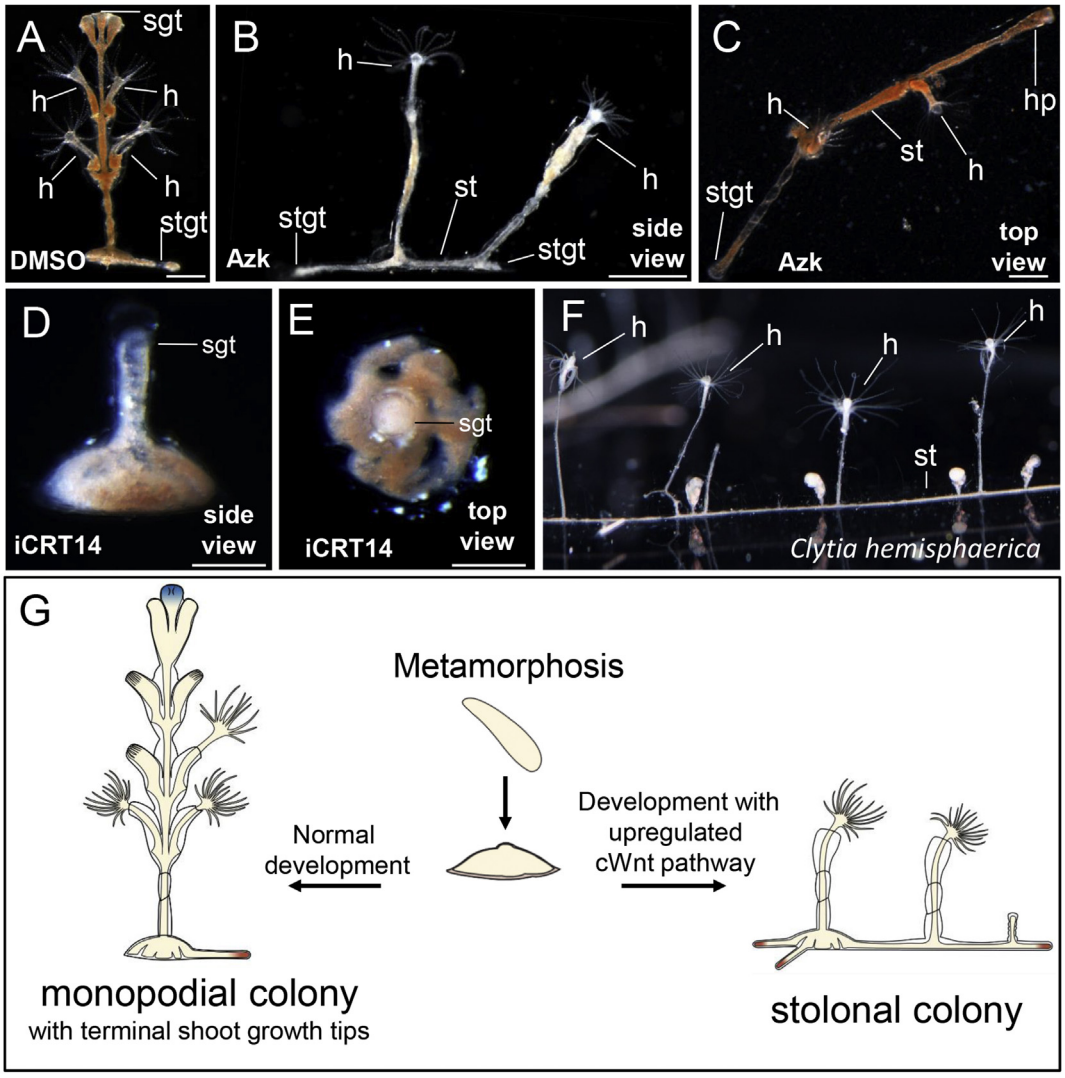

Fig. 4. Modulation of cWnt signaling is responsible for the transformation of the spatial organization of hydroid colony. (A) Intact primary colony of *Dynamena*. (B, C) Hyperactivation of cWnt signaling leads to formation of stolonal colony. (D, E) Arrested development of the shoot growth tip in the result of long-term treatment by 5 μM iCRT14. (F) Stolonal colony of *Clytia hemisphaerica* (image provided by M. Lechable and A. Jan). (G) Scheme of the involvement of cWnt signaling into the colony type specification occurring during planula metamorphosis. blue – shoot growth tip, red – stolon growth tip. hp, hydranth primordium; h, hydranths; sgt, shoot growth tip; stgt, stolon growth tip; st, stolon. Scale bar: (A–C), 300 μM, (D, E) 150 μM.

stolon growth tip. iCRT14 treatment changes spatial expression pattern of almost all genes under the study and decreases the level of their expression as well, and only the level of *Dp-tcf* expression decreases insignificantly (Figs. 3G, S2F,S3). Intriguingly, after the treatment of the primary colonies for 7 days with 5 μM iCRT14 and the subsequent washout of the chemical, we observed differentiation of stolon growth tips instead of shoot growth tips, and, consequently, stolon formation instead of shoot formation (Fig. 5). Stolons were identified by their ability to attach and spread over the substrate. Experimentally provoked differentiation of stolon growth tips instead of shoot growth tips by the modulation of cWnt signaling activity clearly demonstrates that this pathway has a fundamental relevance to a patterning system that controls the hydrozoan body plan during development rather than just regulating head formation (Duffy et al., 2010; Hobmayer et al., 2000). We propose a model of the colony spatial patterning where specification of all structures (i.e. hydrants, shoot growth tips, stolon growth tips and subsequently stolons) depends on the cWnt signaling activity, but in a dose-dependent manner. This means that specific levels of cWnt activity must be maintained in the different areas of the colony. It is important that strong downregulation of cWnt suppresses formation of all colony structures. We still have to explore how these levels of cWnt activity are established and maintained across the colony body.

4. Conclusions

We have shown that experimental manipulation of cWnt signaling can lead to changes in the body plan and organization of the colonial cnidarian *Dynamena*. Using a functional approach, we generated phenotypes which recapitulate (at least on the level of gross morphology) the structures of other colonial hydrozoan species existing in nature, suggesting that modulations of cWnt signaling could have played a role in the evolutionary diversification of body plans in colonial cnidarians. Our results are in agreement with hypotheses suggesting that relatively minor changes in the activity of signaling pathways or regions of gene expression could result in dramatic changes in developmental trajectories (Martín-Durán et al., 2016), transitions between different morphologies of adaptive traits (Bhullar et al., 2015; Parsons et al., 2014) or even body plans (Duffy, 2011). On the other hand, our discovery of the involvement of cWnt signaling in the control of branching of the hydrozoan colonies offers an intriguing possibility that cWnt might have been the ancestral

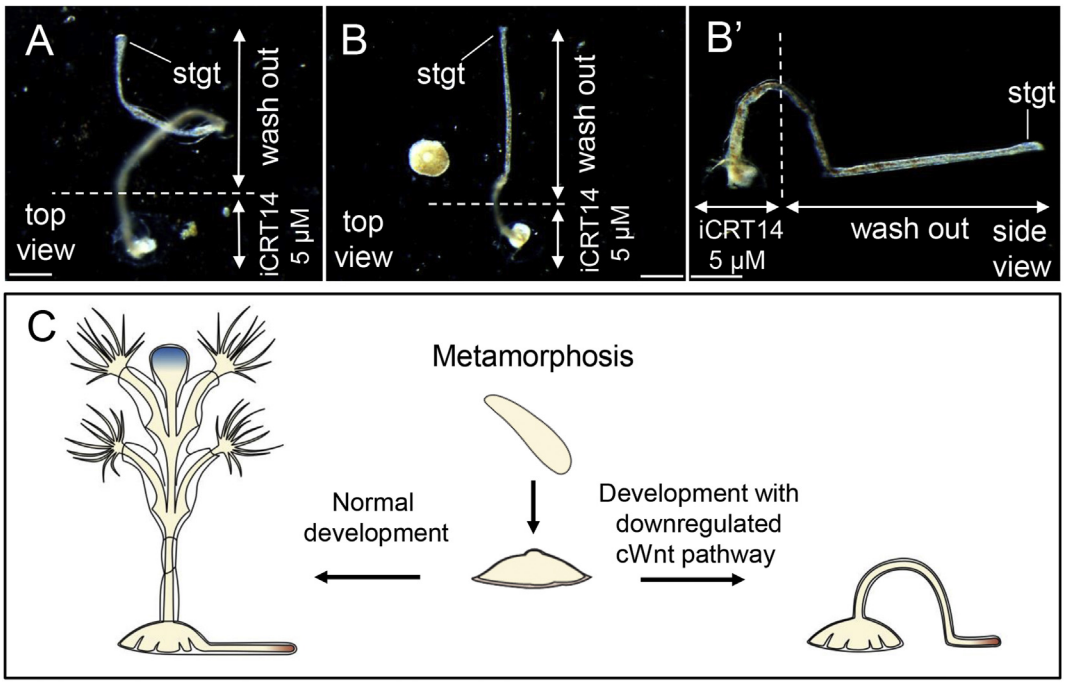

Fig. 5. Pulsed inhibition of cWnt signaling activity led to differentiation of stolon growth tips instead of shoot growth tips. (A-B′) A pulse of 5 μM iCRT14 treatment leads to differentiation of stolon growth tips instead of shoot growth tips. (C) Scheme of the involvement of cWnt signaling into the growing tip fate specification. blue – shoot growth tip, red – stolon growth tip. Scale bar: (A-B′) 300 μM.

regulator of the branching morphogenesis in general. Branching of multiple vertebrate organs such as lung, kidney, salivary gland and mammary gland is cWnt-dependent (Warburton et al., 2005). Intriguingly, the cWnt hyperactivation suppresses branching not only in hydrozoan colony, but in the mammalian embryos as well, e.g. during the lung and lacrimal gland morphogenesis (Dean et al., 2005). A wider phylogenetic sampling and careful functional analyses of branching outside vertebrates and *Drosophila* will be required in order to understand whether this regulatory feature is ancestral or convergent.

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Authors' contributions

T.S.B. and S.V.K. designed the experiments. T.S.B., D.M.K., A.A.V., Y.A.K. and S.V.K. performed the experiments. T.S.B., I.A.K., Y.A.K. and S.V.K. analysed data. S.V.K. developed the concept and wrote the first version of the manuscript. All authors contributed to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.019.

References

Bookout, A.L., Cummins, C.L., Mangelsdorf, D.J., Pesola, J.M., Kramer, M.F., 2005. High-throughput real-time quantitative reverse transcription PCR. Curr. Protoc. Mol. Biol. 15 (8), 1–15. https://doi.org/10.1002/0471142727.mb1508s71.

Banghart, M.R., Volgraf, M., Trauner, D., 2006. Engineering light-gated ion channels. Biochemistry 45, 15129–15141. https://doi.org/10.1021/bi0618058.

Beloussov, L.V., Badenko, L.A., Katchurin, A.L., Kurilo, L.F., 1972. Cell movements in morphogenesis of hydroid polypes. J. Embryol. Exp. Morphol. 27, 317–337.

Berking, S., Hesse, M., Herrmann, K., 2002. A shoot meristem-like organ in animals; monopodial and sympodial growth in Hydrozoa. Int. J. Dev. Biol. 46, 301–308.

Bhullar, B.A.S., Morris, Z.S., Sefton, E.M., Tok, A., Tokita, M., Namkoong, B., Camacho, J., Burnham, D.A., Abzhanov, A., 2015. A molecular mechanism for the origin of a key evolutionary innovation, the bird beak and palate, revealed by an integrative approach to major transitions in vertebrate history. Evolution 69, 1665–1677. https://doi.org/10.1111/evo.12684.

Bielen, H., Oberleitner, S., Marcellini, S., Gee, L., Lemaire, P., Bode, H.R., Rupp, R., Technau, U., 2007. Divergent functions of two ancient Hydra Brachyury paralogues suggest specific roles for their C-terminal domains in tissue fate induction. Development 134, 4187–4197. https://doi.org/10.1242/dev.010173.

Cartwright, P., 2004. The development and evolution of hydrozoan polyp and colony form. Hydrobiologia 530/531, 309–317. https://doi.org/10.1007/s10750-004-2639-7.

Dean, C.H., Miller, L.A.D., Smith, A.N., Dufort, D., Lang, R.A., Niswander, L.A., 2005. Canonical Wnt signaling negatively regulates branching morphogenesis of the lung and lacrimal gland. Dev. Biol. 286, 270–286. https://doi.org/10.1016/j.ydbio.2005.07.034.

Duffy, D.J., 2011. Modulation of Wnt signaling: a route to speciation? Commun. Integr. Biol. 4, 59–61. https://doi.org/10.4161/cib.4.1.13712.

Duffy, D.J., Plickert, G., Kuenzel, T., Tilmann, W., Frank, U., 2010. Wnt signaling promotes oral but suppresses aboral structures in Hydractinia metamorphosis and regeneration. Development 137, 3057–3066. https://doi.org/10.1242/dev.046631.

Fritzenwanker, J.H., Saina, M., Technau, U., 2004. Analysis of forkhead and snail expression reveals epithelial-mesenchymal transitions during embryonic and larval development of Nematostella vectensis. Dev. Biol. 275, 389–402. https://doi.org/10.1016/j.ydbio.2004.08.014.

Genikhovich, G., Technau, U., 2017. On the evolution of bilaterality. Development 144, 3392–3404. https://doi.org/10.1242/dev.141507.
Genikhovich, G., Technau, U., 2009. In situ hybridization of starlet sea anemone (Nematostella vectensis) embryos, larvae, and polyps. Cold Spring Harb. Protoc. 4. https://doi.org/10.1101/pdb.prot5282.
Hobmayer, B., Rentzsch, F., Kuhn, K., Christoph, M., 2000. WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. Nature 407, 1998–2001. https://doi.org/10.1038/35025063.
Kayal, E., Bentlage, B., Sabrina Pankey, M., Ohdera, A.H., Medina, M., Plachetzki, D.C., Collins, A.G., Ryan, J.F., 2018. Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. BMC Evol. Biol. 18, 1–18. https://doi.org/10.1186/s12862-018-1142-0.
Kosevich, I. a, Fedosov, a E., 2008. Morphogenesis in colonial hydroids: pulsating rudiment splitting. Ontogenez 39, 345–361. https://doi.org/10.1134/S1062360408050044.
Kostyuchenko, R.P., Kozin, V.V., Filippova, N.A., Sorokina, E.V., 2018. *FoxA* expression pattern in two polychaete species, *Alitta virens* and *Platynereis dumerilii* : examination of the conserved key regulator of the gut development from cleavage through larval life, post-larval growth and regeneration. Dev. Dyn. 1–16. https://doi.org/10.1002/dvdy.7.
Kraus, J.E.M., Fredman, D., Wang, W., Khalturin, K., Technau, U., 2015. Adoption of conserved developmental genes in development and origin of the medusa body plan Adoption of conserved developmental genes in development and origin of the medusa body plan. EvoDevo 6, 1–15. https://doi.org/10.1186/s13227-015-0017-3.
Kraus, Y., Aman, A., Technau, U., Genikhovich, G., 2016. Pre-bilaterian origin of the blastoporal axial organizer. Nat. Commun. 7, 1–9. https://doi.org/10.1038/ncomms11694.
Kuhn, A., 1914. Entwicklungsgeschichte und Verwandtschaftsbeziehungen der Hydrozoen: die Hydroiden. In: Ergebnisse Und Fortschritte Der Zoologie, pp. 1–284.
Lanna, E., 2015. Evo-devo of non-bilaterian animals, 300, 284–300.
Leclère, L., Bause, M., Sinigaglia, C., Steger, J., Rentzsch, F., 2016. Development of the aboral domain in Nematostella requires β -catenin and the opposing activities of Six3/6 and Frizzled5/8. Development 143, 1766–1777. https://doi.org/10.1242/dev.120931.
Lengfeld, T., Watanabe, H., Simakov, O., Lindgens, D., Gee, L., Law, L., Schmidt, H.A., Özbek, S., Bode, H., Holstein, T.W., 2009. Multiple Wnts are involved in Hydra organizer formation and regeneration. Dev. Biol. 330, 186–199. https://doi.org/10.1016/j.ydbio.2009.02.004.
Marfenin, N., Margulis, R.J., Majer, E.M., 1995. Morphological variability of the colonial hydroid Dynamena pumila, with classification of found morphotypes. Russ. Acad. Sci. Proc. Zool. Inst. 261, 71–89.
Marlow, H., Matus, D.Q., Martindale, M.Q., 2013. Ectopic activation of the canonical wnt signaling pathway affects ectodermal patterning along the primary axis during larval development in the anthozoan Nematostella vectensis. Dev. Biol. 380, 324–334. https://doi.org/10.1016/j.ydbio.2013.05.022.
Martín-Durán, J.M., Passamaneck, Y.J., Martindale, M.Q., Hejnol, A., 2016. The developmental basis for the recurrent evolution of deuterostomy and protostomy. Nat. Ecol. Evol. 1, 0005. https://doi.org/10.1038/s41559-016-0005.
Martindale, M.Q., Hejnol, A., 2009. A developmental perspective: changes in the position of the blastopore during bilaterian evolution. Dev. Cell 17, 162–174. https://doi.org/10.1016/j.devcel.2009.07.024.
Meinhardt, H., 2012. Modeling pattern formation in hydra: a route to understanding essential steps in development. Int. J. Dev. Biol. 56, 447–462. https://doi.org/10.1387/ijdb.113483hm.
Momose, T., Derelle, R., Houliston, E., 2008. A maternally localised Wnt ligand required for axial patterning in the cnidarian Clytia hemisphaerica. Development 135, 2105–2113. https://doi.org/10.1242/dev.021543.
Nakamura, Y., Tsiairis, C.D., Ozbek, S., Holstein, T.W., 2011. Autoregulatory and repressive inputs localize Hydra Wnt3 to the head organizer. Proc. Natl. Acad. Sci. 108, 9137–9142. https://doi.org/10.1073/pnas.1018109108.
Parsons, K.J., Trent Taylor, A., Powder, K.E., Albertson, R.C., 2014. Wnt signalling underlies the evolution of new phenotypes and craniofacial variability in Lake Malawi cichlids. Nat. Commun. 5, 3629. https://doi.org/10.1038/ncomms4629.
Petersen, C.P., Reddien, P.W., 2009. Wnt signaling and the polarity of the primary body Axis. Cell 139, 1056–1068. https://doi.org/10.1016/j.cell.2009.11.035.
Sanders, S.M., Shcheglovitova, M., Cartwright, P., 2014. Differential gene expression between functionally specialized polyps of the colonial hydrozoan Hydractinia symbiolongicarpus (Phylum Cnidaria). BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-406.
Sanetra, M., Begemann, G., Becker, M., Meyer, A., 2005. Conservation and co-option in developmental programmes: the importance of homology relationships. Front. Zool. 2, 15. https://doi.org/10.1186/1742-9994-2-15.
Seipp, S., Wittig, K., Stiening, B., Böttger, A., Leitz, T., 2006. Metamorphosis of Hydractinia echinata (Cnidaria) is caspase-dependent. Int. J. Dev. Biol. 50, 63–70. https://doi.org/10.1387/ijdb.052012ss.
Sinigaglia, C., Thiel, D., Hejnol, A., Houliston, E., Leclère, L., 2017. A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. Dev. Biol. 434, 15–23. https://doi.org/10.1016/j.ydbio.2017.11.015.
Warburton, D., Bellusci, S., De Langhe, S., Del Moral, P.-M., Fleury, V., Mailleux, A., Tefft, D., Unbekandt, M., Wang, K., Shi, W., 2005. Molecular mechanisms of early lung specification and branching morphogenesis. Pediatr. Res. 57, 26R–37R. https://doi.org/10.1203/01.PDR.0000159570.01327.
Wlkramanayake, A.H., Hong, M., Lee, P.N., Pang, K., Byrum, C.A., Bince, J.M., Xu, R., Martindale, M.Q., 2003. An ancient role for nuclear β-catenin in the evolution of axial polarity and germ layer segregation. Nature 426, 446–450. https://doi.org/10.1038/nature02113.