Bacteria- and temperature-regulated peptides modulate β-catenin signaling in *Hydra*

Jan Taubenheim[a,b], Doris Willoweit-Ohl[b], Mirjam Knop[b], Sören Franzenburg[c], Jinru He[b], Thomas C. G. Bosch[b], and Sebastian Fraune[a,b,1]

[a]Zoology and Organismic Interactions, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany; [b]Zoological Institute, Christian-Albrechts University of Kiel, 24118 Kiel, Germany; and [c]Institute of Clinical Molecular Biology, Christian-Albrechts University of Kiel, 24118 Kiel, Germany

Edited by Margaret J. McFall-Ngai, University of Hawaii at Manoa, Honolulu, HI, and approved July 21, 2020 (received for review June 9, 2020)

Animal development has traditionally been viewed as an autonomous process directed by the host genome. But, in many animals, biotic and abiotic cues, like temperature and bacterial colonizers, provide signals for multiple developmental steps. *Hydra* offers unique features to encode these complex interactions of developmental processes with biotic and abiotic factors, and we used it here to investigate the impact of bacterial colonizers and temperature on the pattern formation process. In *Hydra*, formation of the head organizer involves the canonical Wnt pathway. Treatment with alsterpaullone (ALP) results in acquiring characteristics of the head organizer in the body column. Intriguingly, germfree *Hydra* polyps are significantly more sensitive to ALP compared to control polyps. In addition to microbes, β-catenin–dependent pattern formation is also affected by temperature. Gene expression analyses led to the identification of two small secreted peptides, named Eco1 and Eco2, being up-regulated in the response to both *Curvibacter* sp., the main bacterial colonizer of *Hydra*, and low temperatures. Loss-of-function experiments revealed that Eco peptides are involved in the regulation of pattern formation and have an antagonistic function to Wnt signaling in *Hydra*.

Eco-Evo-Devo | host–microbe interaction | orphan gene | phenotypic plasticity | Wnt signaling

Organisms develop in a specific environment, which is recognized and integrated into developmental programs influencing the phenotype and fitness of an individual. Thereby, environmental factors influencing the developmental processes are very diverse, like temperature (1), oxygen levels (2), social interaction (3, 4), or the associated microbiome (5). Adjusting the developmental programs to environmental conditions directly translates into the fitness of an individual and has impact on the evolution (6). The field of ecological evolutionary developmental (Eco-Evo-Devo) biology integrates these three factors into a theoretical framework (7, 8).

Numerous studies explore the effect of temperature on phenotypic differences and the impact on developmental processes (9–12). However, whether the effect is caused by a mere altered chemical reaction norm or whether temperature is actively sensed and developmental programs are adjusted accordingly is still under debate. There is evidence for both scenarios (11, 13), and they might not exclude each other.

Similarly, the associated bacteria of an organism have been shown to affect developmental processes of the host (14). They can drive the first cleavage and determine the anterior–posterior orientation of the fertilized egg of nematodes (15), induce the morphogenesis and settlement of tubeworm larva (5), or impact the correct molting event in filarial nematodes (16). In the squid *Euprymna*, bacteria control the development of the ciliated appendages of the light organs (17), and, in vertebrates, they affect the maturation of the gut (18). How microbial signals or environmental cues are received and integrated into the developmental program of the host is only poorly understood. Sensory nerve cells have been shown to recognize several environmental triggers, like temperature in *Caenorhabditis elegans* (19, 20) and nutrients in *Drosophila melanogaster* (21), and are able to alter phenotypic outcomes during development (22, 23). However, it is unclear whether developmental plasticity has common hubs which are triggered by several environmental cues.

The freshwater polyp *Hydra* harbors a stable microbiota within the glycocalyx of the ectodermal epithelium, which is dominated by a main colonizer *Curvibacter* sp. (24, 25). The microbiota is actively maintained by the host (26–28) and is involved in the protection against fungal infection (25). It appears likely that the microbiota has also an influence on the development of *Hydra* as constantly occurring developmental processes such as regulation of body size are prone to environmental cues (29). *Hydra* belongs to the phylum of Cnidaria, the sister group of all bilateria. It has a radial symmetric body plan with only one body axis and two blastodermic layers, the endo- and the ectoderm (30, 31). While the stem cells reside in the body column, differentiated cells migrate into the head and foot region (32–35). The constant proliferation and differentiation of stem cells and the migration of cells from the body column into the extremities necessitate ongoing pattern formation processes. In *Hydra*, pattern formation is mainly controlled by a Wnt-signaling center in the very tip (hypostome region) of the head (36–38). Transplantation of tissue containing the Wnt organizer can induce a secondary axis in recipient polyps, depending on the position of excision and transplantation, and follows a morphogenetic field model of diffusion reaction (39–42). The formation of the organizer

Significance

Within the life span of an individual, animals adapt to their environment by adjusting their developmental programs dynamically. This phenotypic plasticity is highly specific to the lifestyle of an organism and represents a mechanism which may form the forefront of adaption to new environmental conditions. However, the implementation of the mechanism on the molecular level is not well understood. Here, we show that two taxonomic restricted or orphan genes are modulating the Wnt-signaling pathway in *Hydra* and that their expression depends on temperature and bacterial colonization. Our results demonstrate that environmental cues can be linked to developmental processes by the regulation of orphan genes that modulate conserved signaling pathways.

Author contributions: J.T., S. Franzenburg, T.C.G.B., and S. Fraune designed research; J.T., D.W.-O., M.K., S. Franzenburg, and J.H. performed research; J.T., M.K., and S. Fraune analyzed data; J.T., T.C.G.B., and S. Fraune wrote the paper; and D.W.-O., M.K., S. Franzenburg, and S. Fraune made final manuscript corrections.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: fraune@hhu.de.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2010945117/-/DCSupplemental.

First published August 19, 2020.

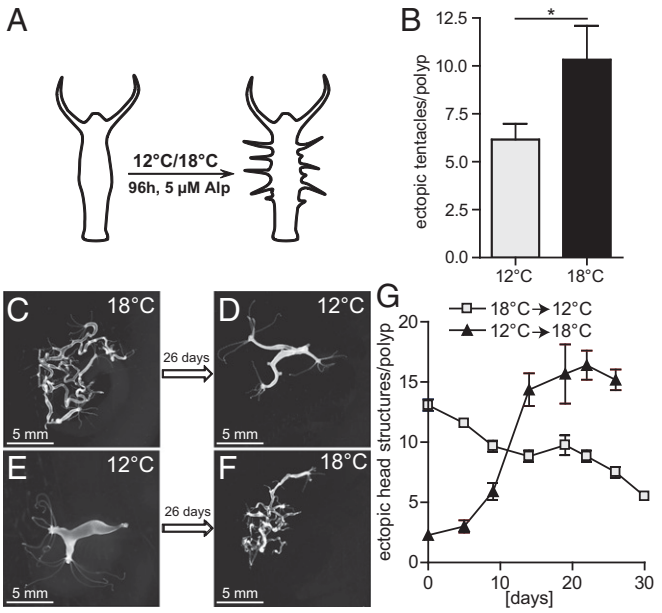

Fig. 1. Wnt signaling is temperature-dependent. (*A*) Animals reared at 12 °C and 18 °C were treated with ALP for 24 h, before assessment of ectopic tentacle formation after 96 h. (*B*) Lower temperature leads to the formation of fewer ectopic tentacles after ALP treatment (*t* test, n =12, $^*P < 0.05$). (*C–F*) Constitutive active Wnt signaling in β-catenin OE animals causes multiple heads/body axes at 18 °C while the severity of the phenotype was subdued when animals were reared at 12 °C. (*G*) The number of heads produced by the β-catenin OE animals is reversible and depends on the rearing temperature, where surrounding temperatures of 12 °C resulted in fewer heads per polyp (n = 10).

integrates not only position information, but is also dependent on the surrounding temperature (39). In addition, ectopic activation of Wnt signaling by the inhibition of the glycogen synthase kinase 3 β (GSK3-β) with alsterpaullone (ALP) induces stem cell differentiation and secondary axis formations (43) by translocating and activating the TCF transcription cofactor β-catenin into the nucleus (38). The unlimited stem cell capacity and the constant pattern formation in *Hydra* endow the organism with extreme plasticity in terms of regeneration, body size adaption to environment, and nonsenescence (44–50). On the molecular level, temperature acts on the Wnt–TGF-β signaling axis, influencing the outcome of developmental decisions such as budding and size regulation of the adult polyp (29). All these processes are reversible, indicating a high degree of plasticity of the developmental programs in *Hydra*. How the environmental cues are received and integrated into the developmental program of the animal remains unknown.

Here, we describe the taxonomically restricted gene (TRG) *eco1* and its paralogue *eco2* to be regulated by long-term temperature and microbiota changes in the freshwater polyp *Hydra*. Changes in the expression of *eco* genes adjust the developmental decisions during pattern formation by interference with the Wnt-signaling pathway, controlling axis stability and continuous stem cell differentiation in *Hydra*.

Results

Temperature and Bacteria Modulate β-Catenin Activity. To consolidate our previous finding that temperature interferes with the Wnt-dependent developmental program in *Hydra* (29), we treated polyps cultured continuously at 12 °C and 18 °C with ALP at 18 °C (Fig. 1*A*). ALP is an inhibitor for GSK3-β and causes an activation of the Wnt-signaling pathway, leading to the formation of ectopic tentacles in *Hydra* (29, 43, 51). The number of ectopic tentacles can be used as a proxy to evaluate Wnt-signaling strength in the animal where higher levels of Wnt signaling leads to a higher number of tentacles. Animals reared at 18 °C formed ∼40% more ectopic tentacles than animals reared at 12 °C (Fig. 1*B*), indicating that lower temperatures decreased Wnt-signaling strength and thus play a role in controlling axis formation and maintenance of the proliferation zone in *Hydra*.

In order to understand how temperature is interfering with the Wnt-signaling pathway, we reared transgenic animals, carrying a constitutively active β-catenin overexpression (OE) (38) construct, at 12 °C and 18 °C. *Hydra* polyps carrying this construct formed multiple secondary axes, and pattern formation was significantly disturbed in these animals (Fig. 1 *C–F*) (38). Transferring these animals from 18 °C to 12 °C rescued this phenotype nearly completely (Fig. 1 *C* and *D*) by reducing the number of heads in these animals over a course of 26 d (Fig. 1*G*). Interestingly, the effect of temperature on axis formation was reversible as animals with few axes reared at 12 °C developed multiple axes within 26 d if cultured at 18 °C (Fig. 1 *E–G*). Temperature thereby had neither an effect on the expression of members of the Wnt-signaling pathway nor the β-catenin OE construct (*SI Appendix*, Fig. S1).

To test whether other environmental factors, such as the associated microbiota, also affect β-catenin–dependent development in *Hydra,* we performed the same ALP treatment on animals with and without associated bacteria (Fig. 2*A*). Germ-free (GF) animals responded nearly four times more to the ectopic activation of Wnt signaling compared to control animals (Fig. 2 *B–D*). In a second experiment, we tested whether recolonization (conventionalizing) of the GF animals reduced the increased tentacle formation after ALP treatment and found a significant mitigation of the ALP effect in these animals (*SI Appendix*, Fig. S2 and Table S1). The observations indicate that not only higher temperatures but also loss of host-associated bacteria increase the Wnt signaling in *Hydra* and affect maintenance of the proliferation zone along the body column.

Both Temperature and Bacteria Influence the Expression of *eco1* and *eco2*. To elucidate the underlying molecular mechanism of the environment–development interaction, we compared differentially

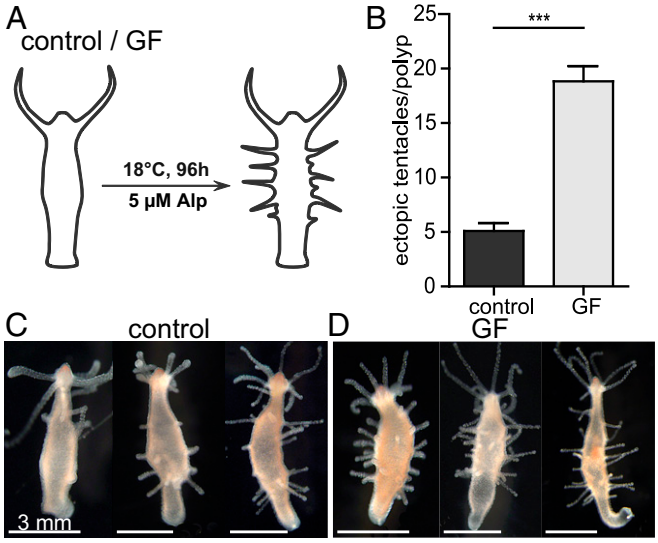

Fig. 2. Wnt signaling is dependent on microbial colonization. (*A*) Animals were ALP-treated for 24 h and the number of generated ectopic tentacles was assessed 96 h after treatment, comparing the treatment outcome of GF and normally colonized animals (control). (*B–D*) Ectopic tentacle formation is increased when colonizing bacteria were removed, suggesting a role of microbial colonization in pattern formation in *Hydra* (Mann–Whitney *U* test, n = 58, $^{***}P < 0.0001$).

regulated genes of a previous microarray study comparing GF to control animals (52) with a recent RNA-seq dataset (29), which compared the transcriptome of animals reared at 12 °C and 18 °C. Both sets of differentially regulated genes overlapped in 55 contigs (Fig. 3*A* and Dataset S1). Within this overlap, 18 genes were regulated in the same direction upon lower rearing temperature and removed bacterial colonization (of these 14 [25.45%] down, four [7.27%] up). The rest of the genes (37 in total, 67.27%) showed contrary regulation in both conditions (19 [34.55%] down at lower rearing temperature, 18 [32.73%] up at lower rearing temperature) (Dataset S1). As low temperature and removal of bacterial colonizers resulted in contrary responses to ALP, we searched for genes with contradicting gene expression in this dataset. We ranked the genes according to their mean expression, fold change, and significance level and excluded metabolic genes for further analysis (see *Materials and Methods* for details of candidate gene selection). The contig 18166 was ranked highest in this analysis and showed highest differential gene regulation in the

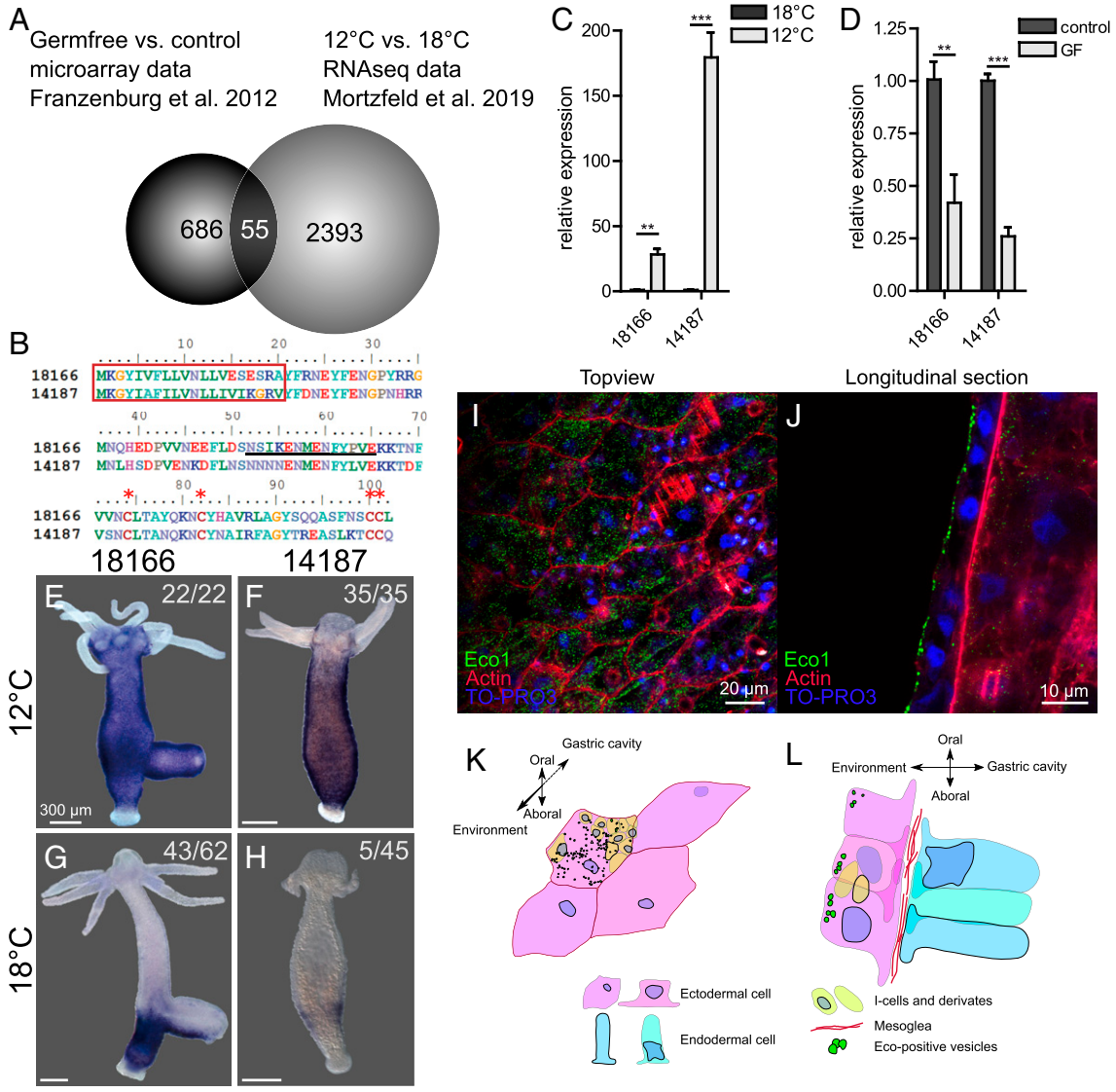

Fig. 3. Two TRGs, expressed in the ectoderm, are potential recognition signals for environmental changes. (*A*) Reanalysis of microarray data, comparing GF animals with colonized ones, and transcriptomic data, comparing animals at different rearing temperatures, revealed two candidate genes with common regulation upon environmental changes: *eco1* and *eco2* (Dataset S1). (*B*) Sequence analysis of the candidate genes revealed a paralogous relationship between the two genes and a secretion signal peptide (red square), without any domain structure. Red asterisk mark cystein residues which indicate formation of cystein bridges. (*C* and *D*) qRT-PCR assessment of *eco* gene expression in GF animals and upon temperature shift confirms down-regulation of *eco1* and *eco2* expression at higher rearing temperatures ($n = 3$, two-way ANOVA, Bonferroni posttests, $^{**}P < 0.01$, $^{***}P < 0.001$) and in disturbed microbiome conditions ($n = 3$, two-way ANOVA, Bonferroni posttests, $^{**}P < 0.01$, $^{***}P < 0.001$). (*E–H*) The *eco* genes were expressed in the foot and lower third of the animals at 18 °C rearing temperature, but the expression domain expands to the body column and parts of the head at 12 °C. Numbers of polyps showing a comparable expression pattern as shown in the imaging compared to all treated animals are indicated in the upper corner of each image. (*I* and *J*) Immunohistochemistry with a polyclonal antibody raised against a fragment of Eco1 (underlined in *B*) show the production of the peptides in the ectodermal epithelium and packaging in vesicles localized in the apical part of the cells, which suggests a secretion of the peptide. The images are displayed in pseudocolor, green, red, and blue corresponding to Eco1 (Alexa Fluor 488), the actin cytoskeleton (rhodamine-phalloidin, a proxy for the mesoglea), and the cell nuclei (TO-PRO3). The image in *I* represents the view on top of the apical part of the ectodermal epithelial cells while the image in *J* displays a longitudinal section through the ecto- and endodermal epithelium. For a clearer overview and better orientation, we provide schematic representations of the topview (*K*) and the longitudinal section (*L*). References cited in *A* are as follows: Franzenburg et al. 2012 (52) and Mortzfeld et al. 2019 (29).

temperature experiment and was on position 15 in the bacterial dataset among the 55 contigs. Apart from contig 18166, a paralogue contig (14187) was found to be regulated by both temperature and bacteria (position 5 and 18 in the temperature and bacterial dataset, respectively). Both paralogues show high sequence homology (65.69% identity, 77.45% similarity), encode for small peptides with a predicted signal for secretion, and contain four conserved cysteine residues (Fig. 3*B*). For both paralogues, no homologs were detectable by BLAST search outside the taxon *Hydra*, indicating that these genes represent TRGs (53, 54).

We tested the expression of both paralogues upon temperature (Fig. 3*C*) and bacterial cues (Fig. 3*D*) via qRT-PCR and observed a significant up-regulation for both genes at low temperature (Fig. 3*C*) and in the presence of bacterial colonizers (Fig. 3*D*), confirming the initial screening result. Notably, the expression response of both genes to temperature changes was much stronger compared to the bacterial response. The spatial expression patterns of both paralogues were analyzed by whole mount in situ hybridizations. At 12 °C, both paralogues were expressed in the ectodermal cell layer along the whole body column, while tentacle and foot tissue showed no expression (Fig. 3 *E* and *F*). The expression at 18 °C was restricted to the foot in the case of 18166 (Fig. 3*G*) and to the lower budding region in the case of 14187 (Fig. 3*H*) or showed no detectable level of expression at all (89%) (*SI Appendix*, Fig. S3). In all cases, the expression domain of 18166 at 18 °C was more expanded than the expression of 14187 (Fig. 3 *G* and *H* and *SI Appendix*, Fig. S3). These results indicate that the expression of both genes is up-regulated due to the expansion of its expression domain from a foot-restricted expression at 18 °C to the expression through the whole body column at 12 °C.

Using a polyclonal antibody, which was generated against a specific peptide encoded by contig 18166 (Fig. 3*B*, underlined sequence; see *SI Appendix*, Fig. S4 for preimmune serum control), we could observe that the peptide is expressed in the ectodermal epithelial layer and localized in small vesicles (Fig. 3 *I* and *K*) accumulating at the apical side of the epithelial cells (Fig. 3 *J* and *L*). This cellular localization suggests that the peptide is secreted at the apical side of the ectodermal cells. Considering their ecological dependence, we termed the genes *eco1* (contig 18166) and *eco2* (contig 14187), respectively.

Expression of *eco1* and *eco2* Response to Environmental Changes within 2 wk. Having confirmed that both genes respond to changes in temperature and bacterial colonization, we assessed the expression of *eco1* and *eco2* over time in GF animals and two controls, conventionalized (conv) animals and wild-type polyps (Fig. 4*A*). While 8 d post recolonization (dpr) the expression levels of *eco1* and *eco2* in conventionalized animals were still equivalent to the levels in GF animals, the expression levels of both genes recovered within the second week, with rising expression levels similar to control animals (Fig. 4*A*). Furthermore, we tested if recolonization with the main colonizer *Curvibacter* sp. alone is sufficient for the regulation of *eco* genes. Analyzing the expression 2 wk after recolonization, we observed a recovery of the expression levels of both genes (Fig. 4*B*), indicating that the specific cross-talk between *Curvibacter* and *Hydra* is sufficient to regulate gene expression of *eco1* and *eco2*.

To get insights into the temporal expression dynamics of *eco1* and *eco2* after temperature change, we transferred animals cultured at 12 °C to 18 °C and vice versa, and monitored the expression over the course of 28 d (Fig. 4*C*). Both genes responded to temperature changes within days, reaching a new stable expression level after around 2 wk (Fig. 4*C*). Thereby, *eco2* showed a higher up-regulation at 12 °C compared to *eco1* (Fig. 4*C*), which might reflect the fact that *eco2* is expressed at a lower level at 18 °C compared to *eco1* (Fig. 3 *G* and *H*).

The fact that both factors, temperature and bacteria, strongly influence the expression of *eco1* and *eco2* raised the question if both factors are interacting with each other. To disentangle both factors, we generated GF animals and maintained them at 18 °C or transferred them to 12 °C (Fig. 4 *D*–*F*). We observed an increase of gene expression in animals transferred to 12 °C independent of the microbiota state of the animals (Fig. 4 *D* and *E*) while the absence of bacteria reduced gene expression independent of the temperature (Fig. 4*F*). The effect of temperature was highly significant in an ANOVA ($P = 8.16E{-}9$), which was corrected for gene variation while the bacteria effect was barely higher than the acceptance level of $\alpha = 0.05$, due to the smaller effect size ($P = 0.0557$). The interaction term of temperature and colonization was not significant ($P = 0.2$), indicating no interaction of temperature and colonization in the regulation of *eco* genes (*SI Appendix*, Table S3). These results suggest independent gene regulation by temperature and microbiota for *eco1* and *eco2*.

In summary, temperature- and bacteria-dependent regulation of the two genes is reversible and reflects a long-term acclimation to both factors, rather than a short-term regulation and an immediate stress response. The timing of expression changes correlates with the reduction of secondary heads in the β-catenin OE animals reared at 12 °C (Fig. 1). Thus, *eco1* and *eco2* might act as effector genes controlling phenotypic plasticity relaying environmental cues directly to developmental pathways.

Eco1 and Eco2 Act as Antagonists to Wnt Signaling. To functionally analyze the role of Eco1 peptides, we designed a hairpin (HP) construct based on the sequence of *eco1* fused to GFP (Fig. 5*A*). We generated two transgenic lines (B5 and B8), which displayed constitutive expression of the HP in the ectodermal epithelial cells. The mosaic nature of genetically modified hatchlings allows for the selection of transgenic and nontransgenic lines, which served as genetically identical control lines (except for the HP construct). On the level of in situ hybridization, the transgenic line B8 showed a dramatically reduced expression level of *eco1* in the whole body column (Fig. 5*B*) in comparison to its control line (Fig. 5*C*). Checking the knock-down rate of *eco1* by qRT-PCR in both lines revealed a strong down-regulation by HP-mediated RNA interference (RNAi). Due to high sequence similarity, the HP-mediated RNAi targeted also *eco2*, leading to similar down-regulation compared to *eco1* in both transgenic lines (Fig. 5*D*). While, at 12 °C, the rate of reduction was between 95 and 100% for both genes, the knock-down rate at 18 °C was between 70 and 80% (Fig. 5*D*).

To test the hypothesis that Eco peptides act as antagonists to Wnt signaling, we treated both transgenic Eco-knockdown (KD) lines with ALP and compared the number of ectopic tentacles to control animals (Fig. 5*E*). We found a significant threefold (B5) and twofold (B8) increase in the number of ectopically formed tentacles in Eco-KD animals, respectively (Fig. 5*F*).

Since the Wnt signaling is instructive for head regeneration, we tested whether Eco peptides are involved in the regeneration process. We cut Eco-KD animals reared at 12 °C and 18 °C in the middle of the body column, to regenerate a head or a foot (*SI Appendix*, Fig. S5*A*). During head regeneration, we observed no difference either in the timing (*SI Appendix*, Fig. S5*B*) or in the number of tentacles which regenerated between the Eco-KD and control animals (*SI Appendix*, Fig. S5*C*). Similarly, foot regeneration was not disturbed in Eco-KD animals (*SI Appendix*, Fig. S5*D*). This result indicates no major role of *eco* genes in the regeneration processes of *Hydra*.

To consolidate the notion of Eco peptides being an antagonist to Wnt signaling, we performed transplantation experiments to measure the head inhibition (HI) potential of Eco-KD animals. In *Hydra,* head activation (HA) and HI are governed by a gradient of an activator and inhibitor, following a model first described by Alan Turing and specified by Alfred Gierer and Hans

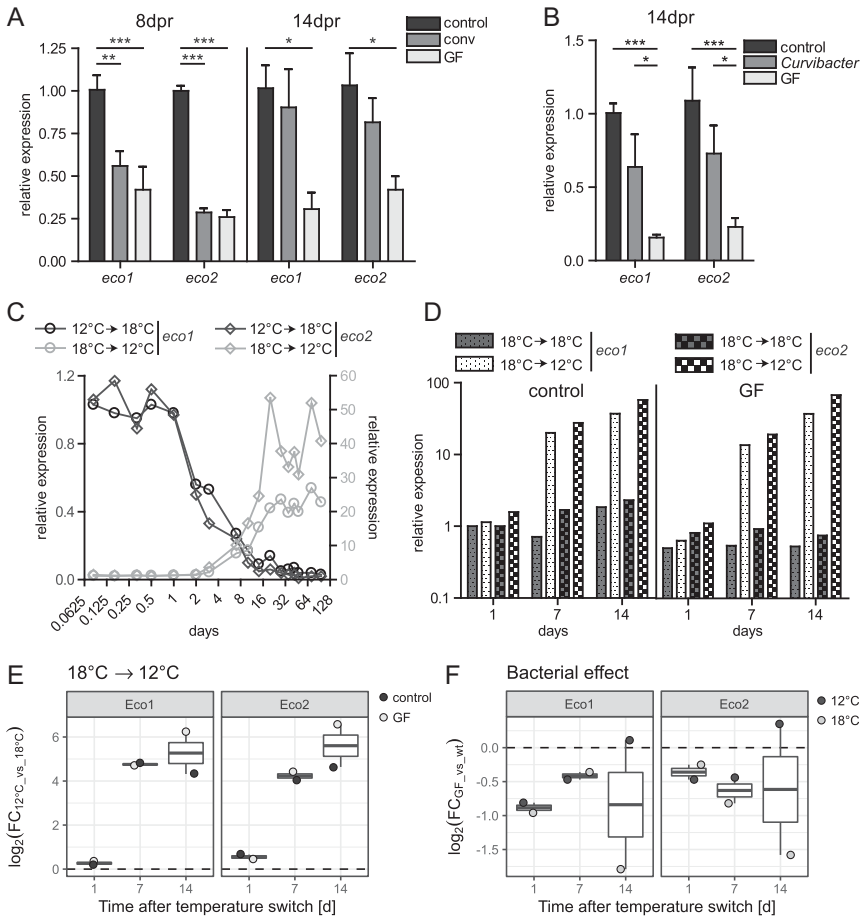

Fig. 4. Expression dynamics of eco genes were a long-term adaptation to changing microbial state or rearing temperatures. (*A*) The eco gene expression was reestablished by recolonization with bacteria after 14 d but did not reach normal levels after only 8 d recolonization ($n = 3$, two-way ANOVA, Bonferroni posttests, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*B*) Recolonization with the main colonizer *Curvibacter* sp. alone was sufficient to alleviate the expression suppression of eco genes after 14 d of colonization ($n = 4$, two-way ANOVA, Bonferroni posttests, $^{*}P < 0.05$, $^{***}P < 0.001$). (*C*) The eco gene expression changes were established within 14 to 16 d upon rearing temperature shifts from 18 °C to 12 °C and vice versa. (*D*) Microbial colonization state and rearing temperature were independent inputs for the expression regulation of eco genes. Gene expression is up-regulated upon temperature shifts from 18 °C to 12 °C, independent of colonization state of the animals. (*E* and *F*) The graphic displays effect size cleaned of overlaying variances. (*E*) The expression of *eco* genes increases within 14 d after reduction of rearing temperature, independent of the colonization status of the genes (ANOVA, $P = 8.16E{-}9$ for temperature, $P = 6.99E{-}6$ for temperature–time interaction [*SI Appendix*, Table S3]). (*F*) Removing bacteria from *Hydra* reduces expression of *eco* genes independent of temperature (ANOVA, $P = 0.0557$ for colonization, $P = 0.20$ for colonization–temperature interaction (*SI Appendix*, Table S3). wt, wild-type; FC, fold change.

Meinhardt (41, 55, 56). The model describes a two component system of molecules, which can explain the head-forming properties of *Hydra's* patterning processes. The idea was experimentally tested by Harry MacWilliams in the 1970s and 1980s, using transplantation techniques (39, 57). In his experiments, he described properties of the head inhibitor and head activator in the animals, using the fact that head near pieces have organizer functionality (40) and can induce a head in the body column of an acceptor polyp. He described two main findings. First, the rate of head induction increased as the site of transplantation was further away from the head of the donor (HI gradient) (39). Second, the potential to form a head decreases with the distance to the head of the excised pieces (HA gradient) (57). We used a similar approach and assessed properties of the HI gradient in the Eco-KD background, by comparing the fraction of heads formed in Eco-KD and control animals (Fig. 5*G*). To this end, we transplanted head near pieces (directly beneath the tentacle ring) from control donor animals into the body column (approximately one-third from the head) of acceptor animals. We chose the site of transplantation (one-third length from the head) as it was reported that head formation was medium to low in this experimental setting (58). If our notion of decreased HI for the knockdown of the *eco* gene expression was true, we expected an increased fraction of heads formed after transplantation of head near tissue into the body column of Eco-KD animals.

We found that the fraction forming heads after transplantation is doubled in Eco-KD animals (31 of 75), compared to control animals (16 of 76), indicating a reduced HI potential (Fig. 5 *G–I*). Together with the ALP experiment, these results demonstrated that Eco peptides antagonize the effects of Wnt signaling. Lastly, we checked whether *eco* gene expression is regulated by the Wnt pathway and can act as a feedback mechanism in the head formation process. We treated animals with different concentrations of ALP (0.2, 1, and 5 μM) for 24 h and measured *eco* expression via qRT-PCR, but could not detect a regulation of the genes (*SI Appendix*, Fig. S6).

Taken together, these results show that the *eco* genes are able to relay environmental signals, like bacterial colonization and temperature, to the Wnt-signaling cascade and by that modulate axis and head formation in *Hydra* according to environmental conditions.

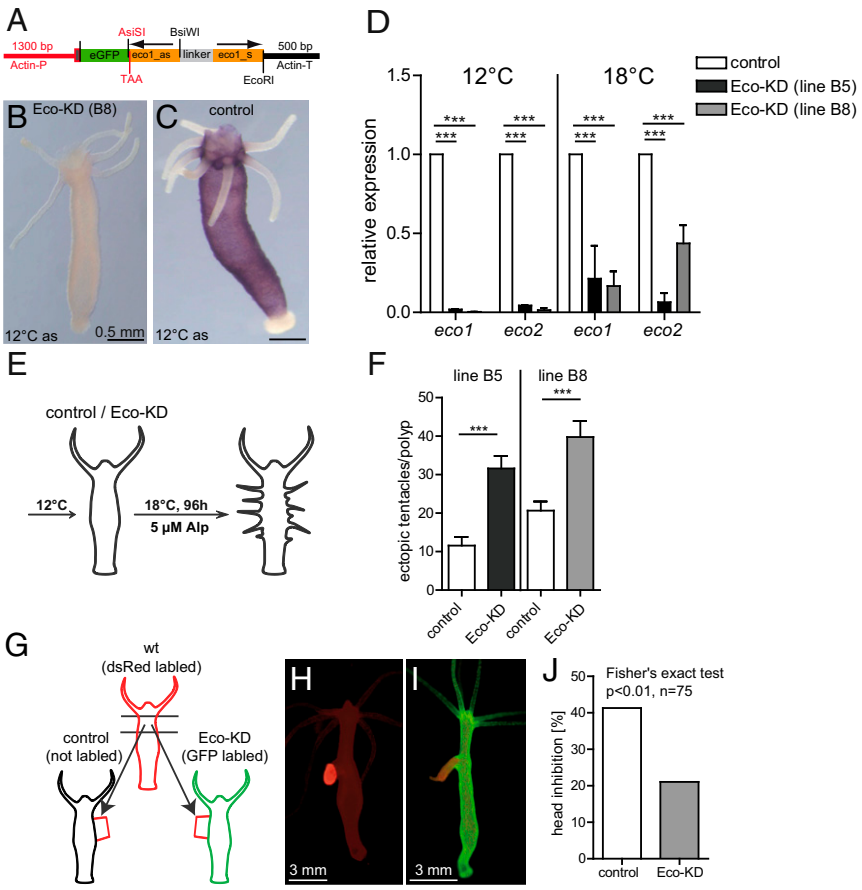

Fig. 5. Knockdown of eco genes results in increased Wnt signaling. (*A*) Eco–HP construct for generation of transgenic *Hydra* (as, antisense; s, sense; TAA, stop codon; P, promoter; T, terminator). (*B* and *C*) Whole mount in situ hybridization of *eco1* expression in Eco-KD (*B*) and control (*C*) animals. (*D*) The *eco1* and *eco2* expression in two transgenic lines (B8 and B5) at 12 °C and 18 °C ($n = 3$, two-way ANOVA, Bonferroni posttests, $^{***}P < 0.001$). (*E* and *F*) Treating Eco1-KD animals with ALP revealed an increased potential to form ectopic tentacles, indicating a higher Wnt-signaling activity (*t* test, $n = 12$, $^{***}P < 0.001$). (*G*) The number of heads formed after transplantation of near head tissue into the body axis of an acceptor polyp serves as a readout for the HI potential, which is governed by the inhibition of the Wnt signaling. (*H* and *I*) Control (unlabeled) and Eco-KD (GFP labeled) animals served as acceptor polyps for head tissues from wild-type (wt) (dsRed labeled) animals to assess the HI potential under disturbed *eco* expression. (*J*) Eco-KD polyps showed a reduced HI potential, indicating an impaired Wnt inhibition in these animals. $n = 75$, $P = 0.0085$, Fisher's exact test. bp, base pairs.

Discussion

Wnt Signaling: An Evolutionarily Conserved Signaling Hub Integrating Environmental Signals to Stem Cell Behavior. The Wnt-signaling pathway most likely evolved in the common ancestor of multicellular animals. Members of the pathway are present in all metazoan animals, but not in fungi, plants, or unicellular eukaryotes, and have regulatory functions in embryogenesis and cell differentiation (59).

In *Hydra*, the Wnt pathway is involved in head formation (36), control of bud formation (37, 60), and the differentiation of stem cells (61, 62). Most Wnt family members are expressed in the tip of the hypostome of *Hydra* (37), and the Wnt pathway has been shown to form the activating agent in the head organizer of *Hydra* (36, 37). Wnt corresponds to the head inducer in the Gierer–Meinhardt model, which describes a self-organizing system of HA and HI and is suitable to explain regeneration and axis formation in *Hydra* (42). In this model, the HI would correspond to a molecule/gene which is activated by Wnt and is inhibiting the Wnt pathway. Thus, such a gene would be expected to be expressed in a graded manner from head to foot. Here, we show that Eco peptides antagonize the Wnt-signaling pathway (Fig. 6) by reducing both ectopic tentacle formation after ALP treatment (Fig. 5*F*) and by transplantation experiments (Fig. 5 *H–J*). However, the expression domain of *eco* genes is restricted to the foot at 18 °C and expands to the head region only, if the animals were reared at 12 °C for at least 14 d (Figs. 3 *E–H* and 4*C*). The expansion of the expression domain of *eco* genes corresponds to a dramatic increase of the gene expression level, which seems not to appear graded in any form (Fig. 3 *C* and *E–H*). Eco peptides do thus not equal the proposed HI of the Gierer–Meinhardt model and therefore add another layer to the Wnt-pathway regulation in *Hydra*.

The properties of the HI in *Hydra* were elaborately determined by transplantation experiments by MacWilliams (39). Inhibitor level changes occurred during regeneration within 6 to 24 h after ablation, indicating that the here-described inhibitor of the Wnt signaling acts on completely different timescales than the immediately responding HI. MacWilliams further noted that temperature changes had an influence on the HI potential of *Hydra* (39). However, these temperature changes were also applied only several hours before transplantation where *eco* expression changes have not occurred (Fig. 4*C*). We therefore argue that Eco peptides do not contribute to the immediate organizing function of Wnt during head formation, but rather are adjusting background levels of Wnt signals throughout the body. This notion is confirmed by the undisturbed regeneration of Eco-KD animals (*SI Appendix*, Fig. S5) and the otherwise normal appearance of the polyps (including regular budding). A regulation of the background

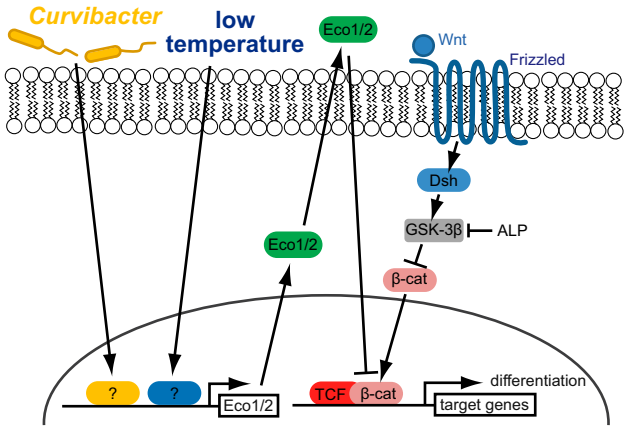

Fig. 6. Eco peptides are environmentally triggered inhibitors of Wnt signaling. Microbial colonization and low temperature are independent inputs for Eco peptides and are able to induce its transcription. Eco peptides are synthesized as small peptides, packed into vesicles and secreted into the extracellular matrix of the ectoderm. By an unknown mechanism, Eco peptides are recognized, and the Wnt signaling is inhibited. The inhibition takes place downstream of the β-catenin (β-cat) stabilization, presumably at the expression of Wnt target genes as Eco expression was able to inhibit activation of the pathway at the GSK-3β level after ALP treatment (see text).

Wnt-signaling strength by Eco peptides seems reasonable because Wnt signaling is important for size regulation in *Hydra* by means of bud initiation (29, 60), and body size is regulated with temperature (29, 49). In addition, the variation of multiple axes in the β-catenin OE animals at different rearing temperatures occurred in the same time frame as *eco* gene regulation upon temperature shift (Fig. 1 *C*–*G*). Altogether, this could imply a function of Eco peptides in adjustment of Wnt-signaling strength as they are regulated with temperature and antagonize Wnt signaling. However, Eco-KD animals showed no obvious size differences in our cultures, which indicates that the temperature–Wnt regulation seems to be more complex and cannot be reduced to Eco signaling alone.

Notably, the proposed HI of the Gierer–Meinhardt model remains enigmatic on the molecular level until today although various candidates have been proposed: for instance, a *Hydra* dickkopf homolog (63, 64), the transcription factor Sp5 (65), or the glycoprotein thrombospondin (66). But none of them seem to resemble all assumed properties of the HI in the Gierer–Meinhardt model. Further, several downstream genes of the Wnt-signaling pathway have been shown to be involved in specific aspects of Wnt signaling: For example, the homeobox gene *rax* is important for tentacle formation (67), PKC signaling seems to be important for head regeneration (68), but not in bud formation (69), while ERK signaling was essential for both processes (68, 69). We thus argue that several levels of Wnt inhibition exist and that the activity of Eco peptides provides one mechanism. Until now, it is unclear how Eco peptides antagonize the Wnt pathway, but we assume that they act downstream of β-catenin, given the following arguments. First, we showed that Eco-KD animals have a higher probability of forming tentacles after ALP treatment but have normal regeneration capability; thus, Rax could be a target transcription factor inhibited upon Eco signaling (67). Secondly, we showed that low temperature is antagonizing the effect of overexpressed β-catenin. As β-catenin was mutated in a way that it was stabilized against APC-dependent degradation (38), we argue that the antagonistic activity is most likely downstream of β-catenin and independent of the APC pathway. Therefore, we predict the presence of an endogenous receptor that binds Eco peptides and activates a signaling cascade antagonizing β-catenin activity.

To date, the inputs of *eco* gene regulation on the molecular level are unknown. We can exclude that Wnt signaling actively regulates *eco* genes, which would be expected if Eco signaling is involved in the immediate head formation process. However, one could speculate on acyl-homoserine-lacton (AHL) as a possible recognition molecule. We previously showed that AHLs produced by *Curvibacter* are quenched by the *Hydra* host to avoid detrimental gene expression of *Curvibacter* on the host (24). This direct interaction indicates an active recognition of AHLs by the host and would mediate a possible route of regulating *eco* gene expression. From the microarray data, we can exclude also an MyD88-mediated *eco* regulation as MyD88-KD animals showed no differences in expression level of *eco* genes (52).

Predicting inputs for the temperature signal is unequally more difficult as, to date, no definite temperature-sensing mechanism has been found in *Hydra*. Transient receptor potential channels (TRPs) are known to sense high and low temperature and have been identified in *Hydra* (70). TRPM3 has also been linked to high temperature sensation (heat shock) in *Hydra* (71), but if similar mechanisms exist for cold temperature or whether they can mediate the long-term effect of *eco* gene expression is unclear to date. *Eco* expression might be regulated by other head-inhibiting substances as MacWilliams has suggested that physical properties of the HI change at different temperatures, resulting in a longer range of action at lower temperatures, which eventually might translate in an activated *eco* gene expression (39).

Increased Wnt signaling causes the stem cells in *Hydra* to differentiate and to lose their potential to self-renew (61, 62). Interestingly, the expression of both Eco peptides is regulated by the presence of *Curvibacter*, the main bacterial colonizer of *Hydra*, and low temperature. Individual pathways may signal both external signals to the promoter of *eco* genes as both factors regulate the expression of both genes independently (Figs. 6 and 4*D*).

Our model suggests that the ratio of recognized Wnt and Eco peptides by an individual cell determines the activation of Wnt-target genes. Thereby, the opposing signaling outcomes of Wnt and Eco would determine the degree of differentiation in the tissue as increased Wnt signaling causes the differentiation of stem cells (61, 62). This process is mediated by Myc1 in interstitial cells, which is directly affected by the increase of Wnt signals due to ALP treatment (62). Given that Eco peptides are able to counteract increased Wnt signals, we thus argue that the *eco* genes integrate environmental signals into the developmental program and thereby promote the stemness of the cells in the body column of *Hydra*.

There are several studies in vertebrates that showed the integration of environmental signals into the Wnt pathway. In zebrafish, the intestinal bacterium *Aeromonas veronii* enhances β-catenin stability after recognition by MyD88, resulting in higher cell proliferation in the intestine (72). In human epithelial cells, the CagA peptide produced by *Helicobacter pylori* activates β-catenin, leading to transcriptional up-regulation of genes implicated in cancer (73, 74). In addition, activation of the aryl hydrocarbon receptor by natural ligands, which are converted from dietary tryptophan and glycosinolates by intestinal microbes (75, 76), results in the degradation of β-catenin (76) and the suppression of intestinal carcinogenesis. We showed another mechanism, how bacterial signals are integrated into the Wnt-signaling pathway, via an orphan peptide not present outside the *Hydra* genus. All described mechanisms are highly specific to each of the different model systems (MyD88, CagA, AhR, Eco1) and thus most likely evolved independently, which highlights the necessity of individual adaptation dependent on the lifestyles to cope with changing environments. However, conserved developmental signaling like the Wnt pathway seems to form signaling hubs to integrate environmental cues. The Wnt-signaling pathway thereby fulfills central tasks in pattern formation, as well as

stem cell regulation in all metazoans, and is clearly an important target to mediate developmental decisions upon environmental changes.

Orphan Genes as Circuit of Environmental Cues to Host Development. With this study, we present taxonomic restricted or orphan genes that are able to modulate the Wnt-signaling pathway. Orphan genes have been found to play important roles by recruiting new energy resources (77, 78), as well as occupying new habitats (79), and often display functions in developmental programs (79, 80), immunity (26, 81), or mediate interaction with the environment (54, 82, 83). Here, we support the notion that species-specific adaptation to the environment is possible through taxonomic restricted genes in a gradual manner. We show that the two *eco* paralogues transmit the current conditions for temperature and microbiota association to the Wnt pathway and modulate the developmental decision.

Eco Peptides Provide an Example for the Eco-Evo-Devo Concept. Animal development has traditionally been viewed as an autonomous process directed by the host genome. In recent years, it got evident that biotic and abiotic cues provide a variety of signals that are integrated into the developmental program. These observations resulted in the Eco-Evo-Devo concept (7, 8). Our results provide further evidences for this concept on different levels. Firstly, our study supports the idea that development is plastic and responsive to abiotic (temperature) and biotic (microbiota) factors as *eco* gene expression is highly dependent on these factors. Secondly, we show that evolution of new traits does not necessarily demand the change in core developmental pathways, but that newly obtained genes can act as modulators of these pathways, mediating a gain of function in developmental programs. Thirdly, we show that evolutionarily young genes, which modulate conserved developmental signaling cascades, can mediate phenotypic plasticity and increase robustness of patterning formation after disturbance.

Materials and Methods

Animal Culture. All experiments were carried out with either wild-type or transgenic *Hydra vulgaris* AEP (*Hydra carnea*) (84, 85), which were cultured in Hydra medium (HM) (0.28 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.5 mM $NaHCO_3$, and 0.08 mM KCO_3) at 18 °C with a 12/12 h light–dark cycle. Animals were fed ad libitum two to three times a week.

Transgenic Animals. We used β-Catenin OE animals described earlier (38) while the Eco-KD animals were generated during this study applying a previously described HP-gene construct approach (52). The full-length eco1 coding sequences served as sense and antisense part for the construct. For details, we refer the reader to *SI Appendix, SI Materials and Methods*.

Antibiotics and Recolonization. GF animals were generated following an antibiotic treatment with a mixture of ampicillin, rifampicin, streptomycin, and neomycin for 2 wk as described earlier (52). For details, we refer the reader to *SI Appendix, SI Materials and Methods*.

ALP Experiment. Animals were fed 1 d prior to the experiment and treated with 5 μM ALP (Sigma-Aldrich) for 24 h to inhibit the GSK3-β and activate the Wnt-signaling pathway at 18 °C, regardless of previous rearing conditions. After treatment, the polyps were washed and incubated in HM before assessment of ectopic tentacle formation under the binoculars 4 d after treatment. To regard any form of the temperature reaction norm, we assessed the number of tentacles per polyp after tentacles were clearly developed so that a further increase in number of tentacles could not be observed, even at later time points.

Temperature Treatment of β-Catenin Animals. β-catenin OE animals were reared at either 18 °C or 12 °C and standard conditions prior to the experiment. Animals were transferred to treatment temperature HM (18 °C → 12 °C; 12 °C → 18 °C) kept as single polyps in a 12-well plate. Head structures of the visually largest animal were assessed at day 0, 5, 9, 14, 19, 22, 26, and 30. Smaller animal fragments, which might have appeared during the experiment, were removed from the cavity.

RNA Extraction, Complementary DNA Generation, and qRT-PCR. Total RNA was extracted using a TRIzol-chloroform–based protocol, with further purification of RNA using the Ambion PureLink RNA Mini Kit (Thermo Scientific). Complementary DNA (cDNA) was generated from the RNA using the First Strand cDNA Synthesis Kit (Fermentas). qPCR was performed using the GoTaq qPCR Master Mix (Promega) in a 7300 real-time PCR system (ABI). For experimental details, we refer the reader to *SI Appendix, SI Materials and Methods*.

In Situ Hybridization. Whole open reading frames of *eco1* and *eco2* were cloned into pGEM-T (Promega). Digoxigenin (DIG)-labeled probes were generated using T7/SP6 transcription start sites and the DIG RNA Labeling Mix kit (Roche) after the manufacturer's instructions. Hybridization was performed as described previously (86). For details of the protocol, please refer to *SI Appendix, SI Materials and Methods*.

Immunohistochemistry. Immunostaining was performed using standard procedures (87). For details of the protocol, please refer to *SI Appendix, SI Materials and Methods*. For imaging, we used a confocal laser scanning microscope (TCS SP1; Leica)

Gene Selection from Transcriptomic Analysis. We defined a gene set from the overlap of differentially expressed genes from a microarray comparing GF vs. recolonized animals and in an RNA-seq experiment comparing 12 °C vs. 18 °C rearing temperature (55 genes in total) (29, 52). For technical details of RNA-seq and microarray analysis, see refs. 29 and 52. This gene set was associated with ranks according to mean expression, *P* value, and fold change for both conditions (decreasing order for fold change and mean expression, increasing order for *P* values). The ranks for each category were summed, to generate a score which was used to sort the genes. The genes Eco1 (contig 18166) and Eco2 (contig 14187) were at position 2 and 7 in this list. We did not consider the first gene in the table (a serine acetyltransferase, contig 18585) as it was predicted to be associated with metabolic changes, which was not the focus of this study. Eco2 was considered only after further investigation of Eco1 and appearing also in a high rank in this list.

Transplantation Experiments. Rings of tissue right beneath the tentacle ring were excised from donor animals and grafted into the ~1/3 of the body axis from head to foot of the acceptor animal, using fishing strings and polypropylene tubes for fixation of the tissues. Grafts were grown together after 2 to 3 h, and fishing strings were removed. The induction of heads was assessed 2 to 3 d posttransplantation using the fluorescent markers and binoculars. Every form of head structure was evaluated as head, including a single tentacle, a hypostome, or a complete hypostome with a tentacle ring.

Data Availability. Datasets for the RNA-sequencing and the microarray are available at the Sequence Read Archive database (SRP133389) (29) and the Gene Expression Omnibus database (GSE32383) (52), respectively. Sequences for Eco1 and Eco2 were deposited under the GenBank accession numbers MT559335 and MT559336. All other study data are included in the article and *SI Appendix*.

ACKNOWLEDGMENTS. We greatly appreciate provision and microinjection of the *Hydra* embryos by Jörg Wittlieb. We further thank Alexander Klimovich for help in the design of the antibodies and help with the confocal microscopy. This work was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft) (CRC1182). T.C.G.B. gratefully appreciates support from the Canadian Institute for Advanced Research.

1. J. K. McGlashan, R.-J. Spencer, J. M. Old, Embryonic communication in the nest: Metabolic responses of reptilian embryos to developmental rates of siblings. *Proc. R. Soc. B Biol. Sci.* **279**, 1709–1715 (2012).
2. V. Callier, H. F. Nijhout, Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 14664–14669 (2011).
3. A. J. Zera, K. C. Tiebel, Brachypterizing effect of group rearing, juvenile hormone III and methoprene in the wing-dimorphic cricket, *Gryllus rubens*. *J. Insect Physiol.* **34**, 489–498 (1988).
4. A. Ogawa, A. Streit, A. Antebi, R. J. Sommer, A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. *Curr. Biol.* **19**, 67–71 (2009).

5. N. J. Shikuma, I. Antoshechkin, J. M. Medeiros, M. Pilhofer, D. K. Newman, Stepwise metamorphosis of the tubeworm *Hydroides elegans* is mediated by a bacterial inducer and MAPK signaling. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 10097–10102 (2016).
6. M. J. West-Eberhard, *Developmental Plasticity and Evolution*, (Oxford University Press, 2003).
7. S. F. Gilbert, T. C. G. Bosch, C. Ledón-Rettig, Eco-evo-devo: Developmental symbiosis and developmental plasticity as evolutionary agents. *Nat. Rev. Genet.* **16**, 611–622 (2015).
8. E. Abouheif *et al*., Eco-evo-devo: The time has come. *Adv. Exp. Med. Biol.* **781**, 107–125 (2014).
9. S. M. Ghosh, N. D. Testa, A. W. Shingleton, Temperature-size rule is mediated by thermal plasticity of critical size in *Drosophila melanogaster*. *Proc. R. Soc. B Biol. Sci.* **280**, 20130174 (2013).
10. D. Atkinson, *Temperature and Organism Size—A Biological Law for Ectotherms?*, (Academic Press, 1994), pp. 1–58.
11. J. F. Gillooly, E. L. Charnov, G. B. West, V. M. Savage, J. H. Brown, Effects of size and temperature on developmental time. *Nature* **417**, 70–73 (2002).
12. C. Bergmann, *Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse*, (Göttinger Studien, 1848).
13. Q. Li, Z. Gong, Cold-sensing regulates *Drosophila* growth through insulin-producing cells. *Nat. Commun.* **6**, 10083 (2015).
14. S. Fraune, T. C. Bosch, Why bacteria matter in animal development and evolution. *BioEssays* **32**, 571–580 (2010).
15. F. Landmann, J. M. Foster, M. L. Michalski, B. E. Slatko, W. Sullivan, Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric posterior localization and AP polarity establishment. *PLoS Negl. Trop. Dis.* **8**, e3096 (2014).
16. F. Landmann, D. Voronin, W. Sullivan, M. J. Taylor, Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after *Wolbachia* depletion from filarial nematodes. *PLoS Pathog.* **7**, e1002351 (2011).
17. M. K. Montgomery, M. McFall-Ngai, Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* **120**, 1719–1729 (1994).
18. F. De Vadder *et al*., Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 6458–6463 (2018).
19. E. M. Hedgecock, R. L. Russell, Normal and mutant thermotaxis in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4061–4065 (1975).
20. J. E. Kammenga *et al*., A *Caenorhabditis elegans* wild type defies the temperature-size rule owing to a single nucleotide polymorphism in tra-3. *PLoS Genet.* **3**, e34 (2007).
21. N. Okamoto, T. Nishimura, Signaling from Glia and cholinergic neurons controls nutrient-dependent production of an insulin-like peptide for *Drosophila* body growth. *Dev. Cell* **35**, 295–310 (2015).
22. M. Fujiwara, P. Sengupta, S. L. McIntire, Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. *Neuron* **36**, 1091–1102 (2002).
23. A. Sawala, A. P. Gould, The sex of specific neurons controls female body growth in *Drosophila*. *PLoS Biol.* **15**, e2002252 (2017).
24. C. Pietschke *et al*., Host modification of a bacterial quorum-sensing signal induces a phenotypic switch in bacterial symbionts. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E8488–E8497 (2017).
25. S. Fraune *et al*., Bacteria-bacteria interactions within the microbiota of the ancestral metazoan *Hydra* contribute to fungal resistance. *ISME J.* **9**, 1543–1556 (2015).
26. S. Franzenburg *et al*., Distinct antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E3730–E3738 (2013).
27. B. M. Mortzfeld, J. Taubenheim, S. Fraune, A. V. Klimovich, T. C. G. Bosch, Stem cell transcription factor FoxO controls microbiome resilience in *Hydra*. *Front. Microbiol.* **9**, 629 (2018).
28. S. Fraune, T. C. G. Bosch, Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 13146–13151 (2007).
29. B. M. Mortzfeld *et al*., Temperature and insulin signaling regulate body size in *Hydra* by the Wnt and TGF-beta pathways. *Nat. Commun.* **10**, 3257 (2019).
30. T. C. G. Bosch, Why polyps regenerate and we don't: Towards a cellular and molecular framework for *Hydra* regeneration. *Dev. Biol.* **303**, 421–433 (2007).
31. K. G. Field *et al*., Molecular phylogeny of the animal kingdom. *Science* **239**, 748–753 (1988).
32. R. D. Campbell, Tissue dynamics of steady state growth in *Hydra littoralis*. I. Patterns of cell division. *Dev. Biol.* **15**, 487–502 (1967).
33. R. D. Campbell, Tissue dynamics of steady state growth in *Hydra littoralis*. II. Patterns of tissue movement. *J. Morphol.* **121**, 19–28 (1967).
34. R. D. Campbell, Tissue dynamics of steady state growth in *Hydra littoralis*. III. Behavior of specific cell types during tissue movements. *J. Exp. Zool.* **164**, 379–391 (1967).
35. T. W. Holstein, E. Hobmayer, C. N. David, Pattern of epithelial cell cycling in *Hydra*. *Dev. Biol.* **148**, 602–611 (1991).
36. B. Hobmayer *et al*., WNT signalling molecules act in axis formation in the diploblastic metazoan *Hydra*. *Nature* **407**, 186–189 (2000).
37. T. Lengfeld *et al*., Multiple Wnts are involved in *Hydra* organizer formation and regeneration. *Dev. Biol.* **330**, 186–199 (2009).
38. L. Gee *et al*., Beta-catenin plays a central role in setting up the head organizer in *Hydra*. *Dev. Biol.* **340**, 116–124 (2010).
39. H. K. MacWilliams, *Hydra* transplantation phenomena and the mechanism of *Hydra* head regeneration. I. Properties of the head inhibition. *Dev. Biol.* **96**, 217–238 (1983).
40. E. N. Browne, The production of new hydranths in *Hydra* by the insertion of small grafts. *J. Exp. Zool.* **7**, 1–23 (1909).
41. A. Turing, The chemical basis of morphogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **237**, 37–72 (1952).
42. A. Gierer, H. Meinhardt, A theory of biological pattern formation. *Kybernetik* **12**, 30–39 (1972).
43. M. Broun, L. Gee, B. Reinhardt, H. R. Bode, Formation of the head organizer in *Hydra* involves the canonical Wnt pathway. *Development* **132**, 2907–2916 (2005).
44. A. Klimovich *et al*., Non-senescent *Hydra* tolerates severe disturbances in the nuclear lamina. *Aging (Albany NY)* **10**, 951–972 (2018).
45. T. C. G. Bosch *et al*., How do environmental factors influence life cycles and development? An experimental framework for early-diverging metazoans. *BioEssays* **36**, 1185–1194 (2014).
46. A.-M. Boehm, P. Rosenstiel, T. C. G. Bosch, Stem cells and aging from a quasi-immortal point of view. *BioEssays* **35**, 994–1003 (2013).
47. B. Galliot, W. Buzgariu, Q. Schenkelaars, Y. Wenger, Non-developmental dimensions of adult regeneration in *Hydra*. *Int. J. Dev. Biol.* **62**, 373–381 (2018).
48. S. Gufler *et al*., β-Catenin acts in a position-independent regeneration response in the simple eumetazoan *Hydra*. *Dev. Biol.* **433**, 310–323 (2018).
49. J. W. Bisbee, Size determination in *Hydra*: The roles of growth and budding. *J. Embryol. Exp. Morphol.* **30**, 1–19 (1973).
50. J. J. Otto, R. D. Campbell, Tissue economics of *Hydra*: Regulation of cell cycle, animal size and development by controlled feeding rates. *J. Cell Sci.* **28**, 117–132 (1977).
51. M. Leost *et al*., Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. *Eur. J. Biochem.* **267**, 5983–5994 (2000).
52. S. Franzenburg *et al*., MyD88-deficient *Hydra* reveal an ancient function of TLR signaling in sensing bacterial colonizers. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 19374–19379 (2012).
53. K. Khalturin, G. Hemmrich, S. Fraune, R. Augustin, T. C. G. Bosch, More than just orphans: Are taxonomically-restricted genes important in evolution? *Trends Genet.* **25**, 404–413 (2009).
54. D. Tautz, T. Domazet-Lošo, The evolutionary origin of orphan genes. *Nat. Rev. Genet.* **12**, 692–702 (2011).
55. H. Meinhardt, A. Gierer, Applications of a theory of biological pattern formation based on lateral inhibition. *J. Cell Sci.* **15**, 321–346 (1974).
56. M. J. Greber, C. N. David, T. W. Holstein, A quantitative method for separation of living *Hydra* cells. *Rouxs Arch. Dev. Biol.* **201**, 296–300 (1992).
57. H. K. MacWilliams, *Hydra* transplantation phenomena and the mechanism of *Hydra* head regeneration. II. Properties of the head activation. *Dev. Biol.* **96**, 239–257 (1983).
58. J. Takano, T. Sugiyama, Genetic analysis of developmental mechanisms in *Hydra*. VIII. Head-activation and head-inhibition potentials of a slow-budding strain (L4). *J. Embryol. Exp. Morphol.* **78**, 141–168 (1983).
59. T. W. Holstein, The evolution of the Wnt pathway. *Cold Spring Harb. Perspect. Biol.* **4**, a007922 (2012).
60. H. Watanabe *et al*., Nodal signalling determines biradial asymmetry in *Hydra*. *Nature* **515**, 112–115 (2014).
61. K. Khalturin *et al*., Transgenic stem cells in *Hydra* reveal an early evolutionary origin for key elements controlling self-renewal and differentiation. *Dev. Biol.* **309**, 32–44 (2007).
62. M. Hartl *et al*., Differential regulation of myc homologs by Wnt/β-catenin signaling in the early metazoan *Hydra*. *FEBS J.* **286**, 2295–2310 (2019).
63. C. Guder *et al*., An ancient Wnt-Dickkopf antagonism in *Hydra*. *Development* **133**, 901–911 (2006).
64. R. Augustin *et al*., Dickkopf related genes are components of the positional value gradient in *Hydra*. *Dev. Biol.* **296**, 62–70 (2006).
65. M. C. Vogg *et al*., An evolutionarily-conserved Wnt3/β-catenin/Sp5 feedback loop restricts head organizer activity in *Hydra*. *Nat. Commun.* **10**, 312 (2019).
66. M. Lommel *et al*., *Hydra* mesoglea proteome identifies thrombospondin as a conserved component active in head organizer restriction. *Sci. Rep.* **8**, 11753 (2018).
67. P. C. Reddy *et al*., Molecular signature of an ancient organizer regulated by Wnt/β-catenin signalling during primary body axis patterning in *Hydra*. *Commun. Biol.* **2**, 434 (2019).
68. M. Cardenas *et al*., Selective protein kinase inhibitors block head-specific differentiation in *Hydra*. *Cell. Signal.* **12**, 649–658 (2000).
69. Y. Fabila, L. Navarro, T. Fujisawa, H. R. Bode, L. M. Salgado, Selective inhibition of protein kinases blocks the formation of a new axis, the beginning of budding, in *Hydra*. *Mech. Dev.* **119**, 157–164 (2002).
70. A. Schüler *et al*., The rise and fall of TRP-N, an ancient family of mechanogated ion channels, in metazoa. *Genome Biol. Evol.* **7**, 1713–1727 (2015).
71. V. Malafoglia *et al*., Transient receptor potential melastatin-3 (TRPM3) mediates nociceptive-like responses in *Hydra vulgaris*. *PLoS One* **11**, e0151386 (2016).
72. S. E. Cheesman, J. T. Neal, E. Mittge, B. M. Seredick, K. Guillemin, Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 4570–4577 (2011).
73. A. T. Franco *et al*., Activation of β-catenin by carcinogenic *Helicobacter pylori*. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 10646–10651 (2005).
74. N. Murata-Kamiya *et al*., *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the β-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* **26**, 4617–4626 (2007).
75. S. Heath-Pagliuso *et al*., Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry* **37**, 11508–11515 (1998).

76. K. Kawajiri *et al*., Aryl hydrocarbon receptor suppresses intestinal carcinogenesis in ApcMin/+ mice with natural ligands. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13481–13486 (2009).
77. L. Li *et al*., QQS orphan gene regulates carbon and nitrogen partitioning across species via NF-YC interactions. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 14734–14739 (2015).
78. L. Li *et al*., Identification of the novel protein QQS as a component of the starch metabolic network in *Arabidopsis* leaves. *Plant J.* **58**, 485–498 (2009).
79. M. E. Santos, A. Le Bouquin, A. J. J. Crumière, A. Khila, Taxon-restricted genes at the origin of a novel trait allowing access to a new environment. *Science* **358**, 386–390 (2017).
80. K. Khalturin *et al*., A novel gene family controls species-specific morphological traits in *Hydra*. *PLoS Biol.* **6**, e278 (2008).
81. T. B. Sackton *et al*., Dynamic evolution of the innate immune system in *Drosophila*. *Nat. Genet.* **39**, 1461–1468 (2007).
82. J. K. Colbourne *et al*., The ecoresponsive genome of *Daphnia pulex*. *Science* **331**, 555–561 (2011).
83. C.-H. Kuo, J. C. Kissinger, Consistent and contrasting properties of lineage-specific genes in the apicomplexan parasites *Plasmodium* and *Theileria*. *BMC Evol. Biol.* **8**, 108 (2008).
84. M. Schwentner, T. C. G. Bosch, Revisiting the age, evolutionary history and species level diversity of the genus *Hydra* (Cnidaria: Hydrozoa). *Mol. Phylogenet. Evol.* **91**, 41–55 (2015).
85. G. Hemmrich, B. Anokhin, H. Zacharias, T. C. G. Bosch, Molecular phylogenetics in *Hydra*, a classical model in evolutionary developmental biology. *Mol. Phylogenet. Evol.* **44**, 281–290 (2007).
86. A. Grens, L. Gee, D. A. Fisher, H. R. Bode, CnNK-2, an NK-2 homeobox gene, has a role in patterning the basal end of the axis in *Hydra*. *Dev. Biol.* **180**, 473–488 (1996).
87. U. Engel *et al*., Nowa, a novel protein with minicollagen Cys-rich domains, is involved in nematocyst formation in *Hydra*. *J. Cell Sci.* **115**, 3923–3934 (2002).