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

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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 tube worm 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 glycoalyx 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.

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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 alsterpallone (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 parologue 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. 1A). 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. 1B), 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. 1C–F) (38). Transferring these animals from 18 °C to 12 °C rescued this phenotype nearly completely (Fig. 1C and D) by reducing the number of heads in these animals over a course of 26 d (Fig. 1G). 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. 1E–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. 2A). Germ-free (GF) animals responded nearly four times more to the ectopic activation of Wnt signaling compared to control animals (Fig. 2B–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.

![Fig. 1. Wnt signaling is temperature-dependent.](image)

**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
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. 3A 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. 3B). 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. 3C) and bacterial cues (Fig. 3D) via qRT-PCR and observed a significant up-regulation for both genes at low temperature (Fig. 3C) and in the presence of bacterial colonizers (Fig. 3D), 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. 3G) and to the lower budding region in the case of 14187 (Fig. 3H) 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. 3B, 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. 4A). 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 Curivibacter sp. alone has a similar effect on the regulation of eco1 and eco2 genes. Analyzing the expression 2 wk after recolonization, we observed a recovery of the expression levels of both genes (Fig. 4B), indicating that the specific cross-talk between Curivibacter 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. 4C). Both genes responded to temperature changes within days, reaching a new stable expression level after around 2 wk (Fig. 4C). Thereby, eco2 showed a higher up-regulation at 12 °C compared to eco1 (Fig. 4C), 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 independently of the temperature (Fig. 4F). 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 $a = 0.05$, 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. 5A).

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. 5B) in comparison to its control line (Fig. 5C). 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. 5D). 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. 5D).

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. 5E). We found a significant threefold (B5) and twofold (B8) increase in the number of ectopically formed tentacles in Eco-KD animals, respectively (Fig. 5F).

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. S5A). During head regeneration, we observed no difference either in the timing (SI Appendix, Fig. S5B) or in the number of tentacles which regenerated between the Eco-KD and control animals (SI Appendix, Fig. S5C). Similarly, foot regeneration was not disturbed in Eco-KD animals (SI Appendix, Fig. S5D). 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
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). 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. 5G–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, Table S3). 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. 5F) 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 4C). 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. 4C). 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
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 bud initiation (29, 60), and body size is regulated with temperature expression of Wnt target genes as Eco expression was able to inhibit activation of the pathway at the GSX-3β level after ALP treatment (see text).

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 GSX-3β level after ALP treatment (see text).

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). The 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 equally 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 4D).

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 glycosaminolates 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, Ecol) 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 CaCl2, 0.33 mM MgSO4, 0.5 mM NaHCO3, and 0.08 mM KCO3) 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 γ-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.

**Immunohistochemistry.** Immunostaining was performed using standard procedures (87). For details of the protocol, please refer to SI Appendix, SI Materials and Methods.

**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 18116) and Eco2 (contig 141187) 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 tail of the acceptor animals, 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 (SRP133388) (29) and the Gene Expression Omnibus database (GSE23283) (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.

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