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

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Abstract

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. Here, we used the model animal *Hydra* 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, germ-free *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 upregulated 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*. Our results in an early branching metazoan suggest that the Wnt pathway evolved as a signaling hub integrating environmental signals, like bacterial signals and temperature, into developmental processes and stem cell behavior.

Introduction

Organisms develop in a specific environment, which is recognized and integrated into developmental programs determining the phenotype and fitness of an individual. The concept of Ecological Evolutionary Developmental Biology was introduced [1,2] to highlight the impact of phenotypic and developmental plasticity on evolution [3]. Environmental factors which influence the developmental processes can be very diverse like temperature [4], oxygen levels [5], social interaction [6,7] or the associated microbiome [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 are evidences for both scenarios [13,14] and they might not exclude each other.

Similarly, the associated bacteria of an organism have been shown to affect developmental processes of the host [15]. They can drive the first cleavage and determine the anterior-posterior orientation of the fertilized egg of nematodes [16], induce the morphogenesis and settlement of tubeworm larva [8], or impact the correct molting event in filarial nematodes [17]. In the squid *Euprymna*, bacteria control the development of the ciliated appendages of the light organs [18] and in vertebrates they affect the maturation of the gut [19]. 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 *C. elegans* [20,21] and nutrients in *D. melanogaster* [22] and are able to alter phenotypic outcomes during development [23,24]. 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.* [25,26]. The microbiota is actively maintained by the host [27–29] and is involved in the protection against fungal infection [25]. *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 body column resides the stem cells, 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 necessitates 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 [36,37]. 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 [38–41]. The formation of the organizer

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 GSK3-β kinase with Alsterpaullone (ALP) is inducing stem cell differentiation and secondary axis formations [42] by translocating and activating the transcription factor beta-catenin in the nucleus [37]. The unlimited stem cell capacity and the constant patterning in *Hydra* renders the organism extremely flexible in terms of regeneration, adaptation to a changing environment and escaping aging [43–47]. *Hydra* polyps change their body size under different rearing temperatures or feeding regimes [48,49]. We recently could show that temperature acts on the Wnt-TGF-β signaling axis, which influences the outcome of developmental decisions such as budding and thereby size regulation of the adult polyp [50]. Starvation leads to shrinking of the animals while overfeeding leads to morphological anomalies [49]. All these effects 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 *eco1* and its homolog *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 proliferation in *Hydra.*

Results

Temperature and bacteria modulate beta-catenin activity

To investigate whether temperature interferes with the Wnt-dependent developmental program in *Hydra* we cultured polyps at 12 °C and 18 °C and treated the animals with Alsterpaullone (ALP) (**Figure 1A**).

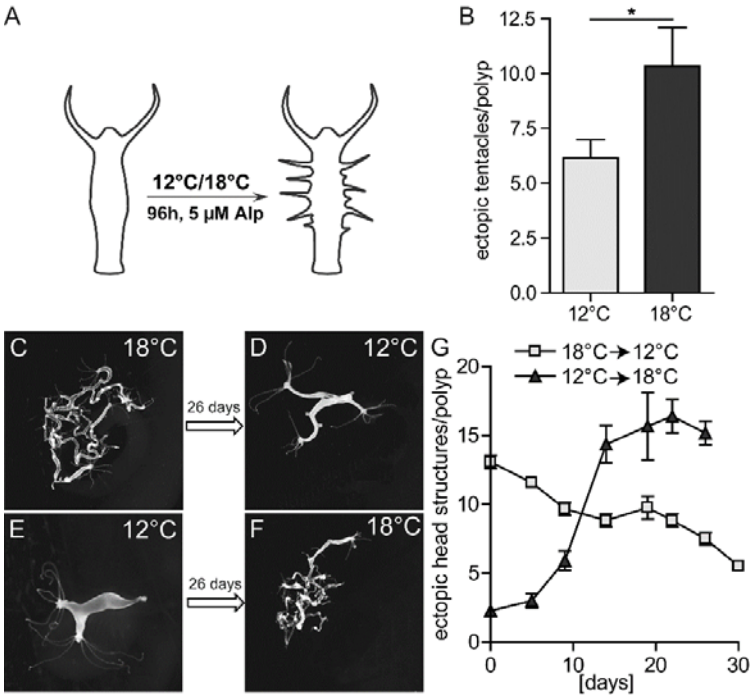

Figure 1: Wnt-signaling is temperature dependent. (A) Animals reared at 12 °C and 18 °C were treated with ALP for 24 h, before assessment of ectopic tentacle formation after 96 h. (B) Lower temperature leads to fewer ectopic tentacle formation after ALP treatment (t-test, n =12, * $p<0.05$). (C-F) Constitutive 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).

ALP is an inhibitor for GSK3-β and causes an activation of the Wnt-signaling pathway [42,50,51]. To estimate the temperature reaction norm we assessed the number of tentacles per polyp after tentacles were clearly developed (~96 h) at both temperatures. Animals reared at 18°C formed ~40% more ectopic tentacles than animals reared at 12°C (**Figure 1B**), indicating that temperature modulates Wnt-signaling and thus plays a role in controlling axis formation and maintenance of the proliferation zone in *Hydra*. These data are in agreement with earlier observations [50]. In order to proof that temperature is interfering with the Wnt-signaling pathway, we reared transgenic animals, carrying a constitutively active β-catenin

overexpression (OE) [37] construct at 12°C and 18°C. *Hydra* polyps carrying this construct formed multiple secondary axis indicating that pattern formation is significantly disturbed in these animals (**Figure 1C-F**) [37]. Transferring these animals from 18°C to 12°C rescued this phenotype nearly completely (**Figure 1C, D**) by reducing the number of heads in these animals over a course of 26 d (**Figure 1G**). Interestingly, the effect of temperature on axis formation was reversible, as animals with few axis reared at 12°C developed multiple axis within 26 d if cultured at 18°C (**Figure 1E, F, G**). Temperature thereby had neither an effect on the expression of members of the Wnt-signaling pathway nor the β-catenin OE construct (**Figure S1**).

To test whether other environmental factors such as the associated microbiota also affects β-catenin dependent development in *Hydra,* we performed the same ALP-treatment on animals with and without associated bacteria (**Figure 2A**). Surprisingly, germfree animals responded nearly four times more to the ectopic activation of Wnt-signaling compared to control animals (**Figure 2B-D**). The observations indicate that not only temperature but also host-associated bacteria interfere with the developmental program of *Hydra* and affect maintenance of the proliferation zone along the body column.

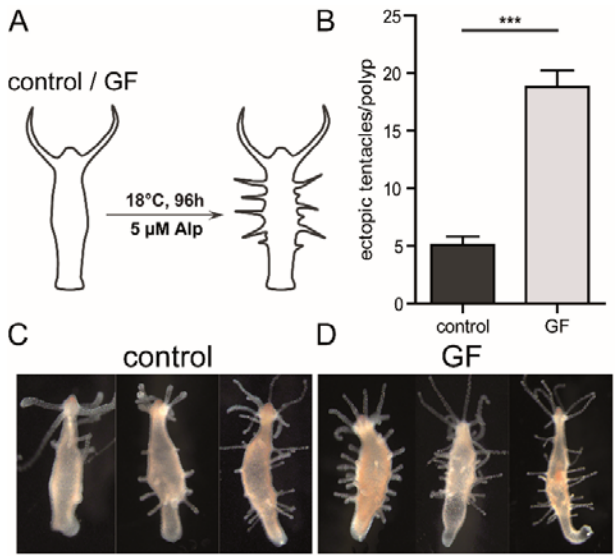

Figure 2: Wnt-signaling is dependent on microbial colonization. (A) Animals were ALP treated for 24 h and number of generated ectopic tentacles were assessed 96 h after treatment, comparing the treatment outcome of germfree (GF) and normally colonized animals (control). (B-D) Ectopic tentacle formation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

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 data set [50], which compared the transcriptome of animals reared at 12°C and 18°C. Both sets of differentially regulated genes overlapped in 55 contigs (**Figure 3A, Table S1**).

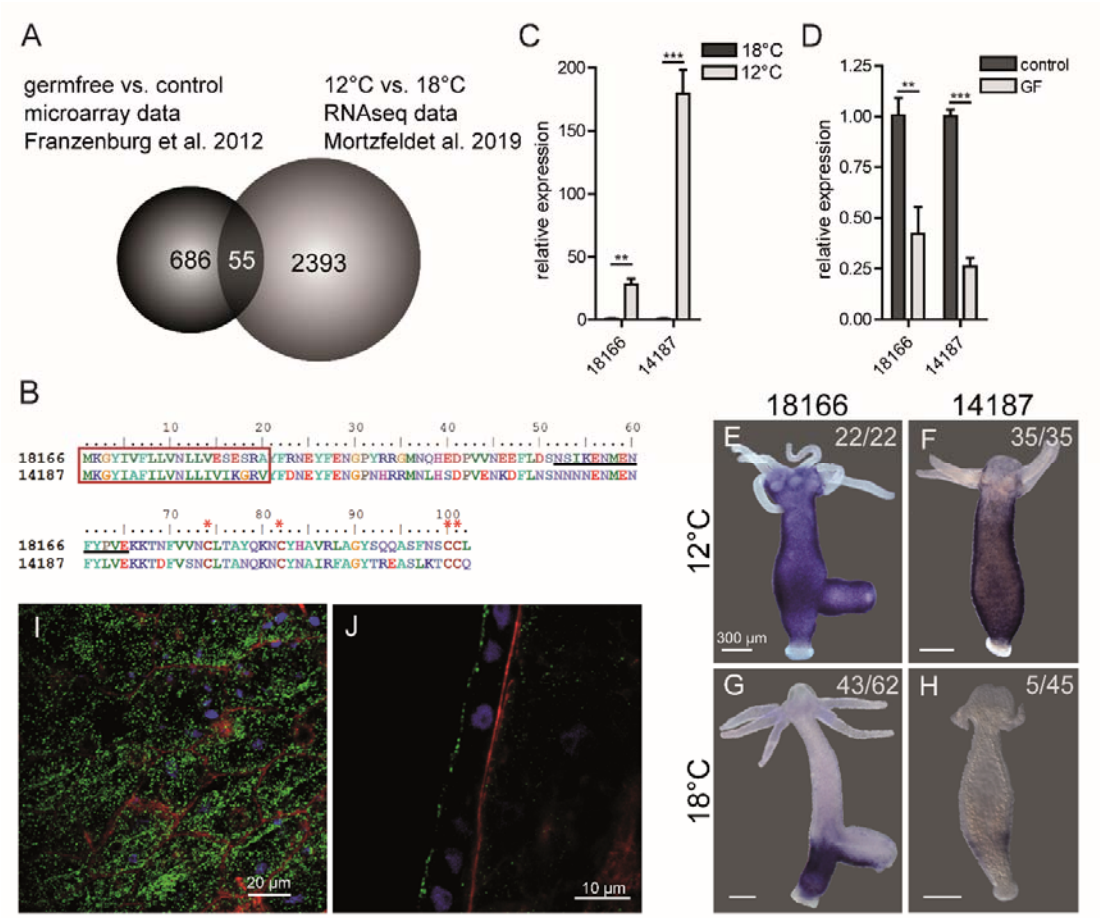

Figure 3: Two TRGs, expressed in the ectoderm, are potential recognition signals for environmental changes. (A) Re-analysis of microarray data, comparing germfree animals with colonized ones, and transcriptomic data, comparing animals at different rearing temperatures, revealed two candidate genes with common regulation upon environmental changes: contig 18166 and 14187. (B) Sequence analysis of the candidate genes revealed a paralogous relationship between the two genes and a secretion signal peptide, without any domain structure. (C-D) qRT-PCR assessment of contig 18166 and 14187 gene expression in germfree animals and upon temperature shift confirms down regulation of both genes 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<0.05, ** p<0.01, *** p<0.001). (E-H) 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 indicate the proportion of polyps showing similar expression pattern). (I-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 contig 18166 showed highest differential gene regulation in the temperature experiment and was on position 15 in the bacterial data set among the 55 contigs. Interestingly, apart from contig 18166 a paralogue contig (14187) was also found to be regulated by both temperature and bacteria (position 5 and 18 in the temperature and bacterial data set, respectively). Both paralogues show high sequence homology, encode for small secreted peptides, and contain four conserved cysteine residues (**Figure 3B**). For both paralogues no homologs are detectable by blast search outside the taxon *Hydra*, indicating that these genes represent taxonomically restricted genes [53,54]. We tested the expression of both paralogues upon temperature (**Figure 3C**) and bacterial cues (**Figure 3D**) via qRT-PCR and observed a significant up-regulation for both genes at low temperature (**Figure 3C**) and in the presence of bacterial colonizers (**Figure 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 pattern of both paralogues were analyzed by whole mount *in-situ* hybridizations. At 12°C, both paralogues are expressed along the whole body column, while tentacle and foot tissue showed no expression (**Figure 3E, F**). The expression at 18°C is restricted to the foot in the case of 18166 (**Figure 3G**) and to the lower budding region in the case of 14187 (**Figure 3H**) or showed no detectable level of expression at all (89%). In all cases the expression domain of 18166 at 18°C was more expanded than the expression of 14187 (**Figure 3G; H**). 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 (**Figure 3B**, underlined sequence), we could observe that the peptide is expressed in the ectodermal epithelial layer, localizes in small vesicles (**Figure 3I**) and accumulates at the apical side of the epithelial cells (**Figure 3J**). This cellular localization suggest that the peptide is secreted at the apical side of the ectodermal cells. To regard 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 two weeks

Having confirmed that both genes respond to changes in temperature and bacterial colonization, we assessed the expression of *eco1* and *eco2* over time in germfree animals and two controls, conventionalized (conv) animals and wildtype polyps (**Figure 4A**).

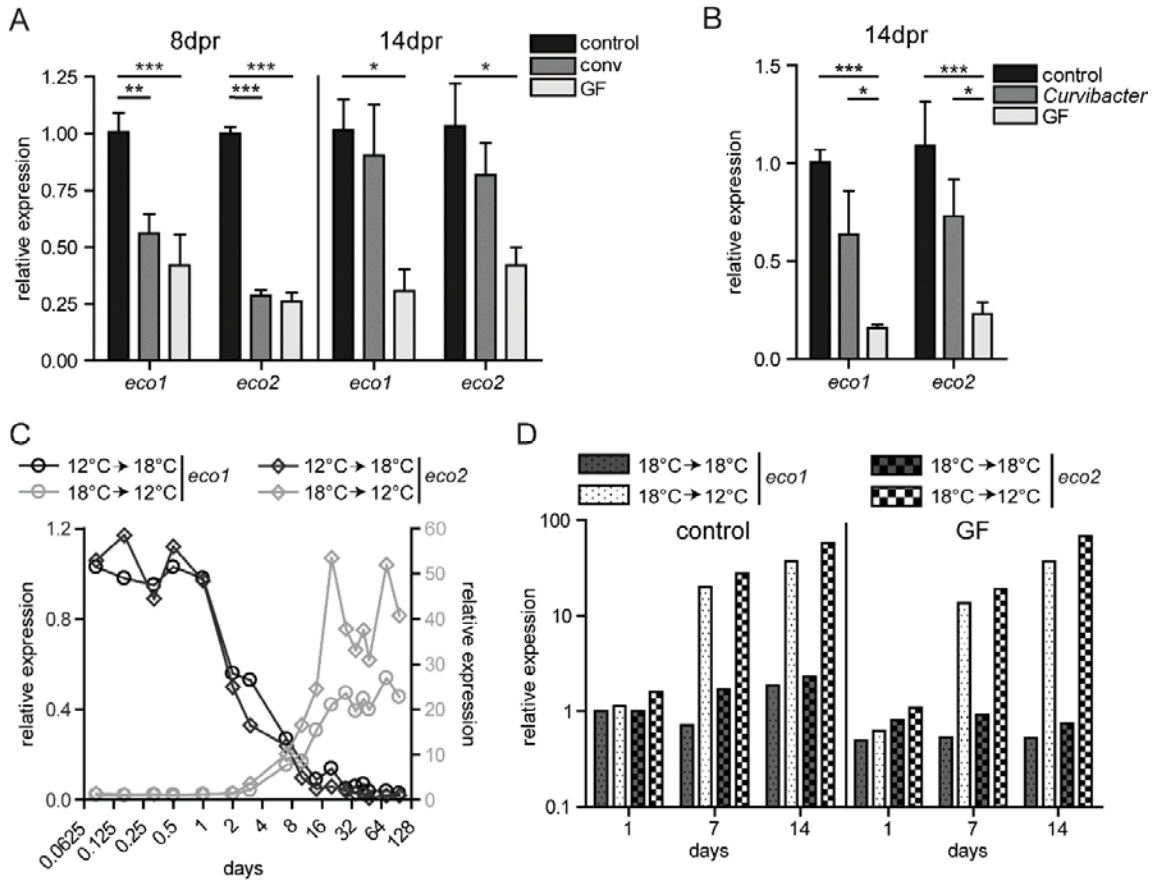

Figure 4: Expression dynamics of eco genes were a long term adaptation to changing microbial state or rearing temperatures. (A) *Eco* gene expression was reestablished by recolonization with bacteria after 14 d, but did not reach normal levels after only 8 d recolonization (n =3, Two-way ANOVA, Bonferroni 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) *Eco* gene expression changes were established within 14-16 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.

While 8 days post recolonization (dpr) the expression level of *eco1* and *eco2* in conventionalized animals were still equivalent to the levels in germfree (GF) animals, the expression level of both genes recovered within the second week, with rising expression levels similar to control animals (**Figure 4A**). Furthermore, we tested if recolonization with the main colonizer *Curvibacter* sp. alone is sufficient for the regulation of *eco* genes. Analyzing the expression two weeks after recolonization we observed a recovery of the expression levels of both genes (**Figure 4B**), indicating that the specific crosstalk 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 days (**Figure 4C**). Both genes responded to temperature changes within days, reaching a new stable expression level after around two weeks (**Figure 4C**). Thereby, *eco2* shows a higher up regulation at 12°C compared to *eco1* (**Figure 4C**), which might reflect the fact that *eco2* is expressed at a lower level at 18°C compared to *eco1* (**Figure 3 G, 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 (**Figure 4D**). We observed an increase of gene expression in animals transferred to 12°C independent of the microbiota state of the animals (**Figure 4D**). 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 are reversible and reflect 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 (**Figure 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 (**Figure 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 non-transgenic lines, which served as genetically identical control lines (except for the hairpin 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 (**Figure 5B**) in comparison to its control line (**Figure 5C**). Checking the knock-down rate of *eco1* by qRT-PCR in both lines revealed a strong down regulation by hairpin-mediated RNAi. Due to high sequence similarity, the hairpin-mediated RNAi targeted also *eco2*, leading to similar down-regulation compared to *eco1* in both transgenic lines (**Figure 5D**). While at 12 °C the rate of reduction was between 95%-100% for both genes, the knock-down rate at 18°C was between 60%-80% (**Figure 5D**).

To test the hypothesis that Eco peptides act as antagonist to Wnt-signaling we treated both transgenic Eco-knockdown (KD) lines with ALP and compared the number of ectopic

tentacles to control animals (**Figure 5E**). We found a significant 3-fold (B5) and 2-fold (B8) increase in number of ectopically formed tentacles in Eco-KD animals, respectively (**Figure 5F**).

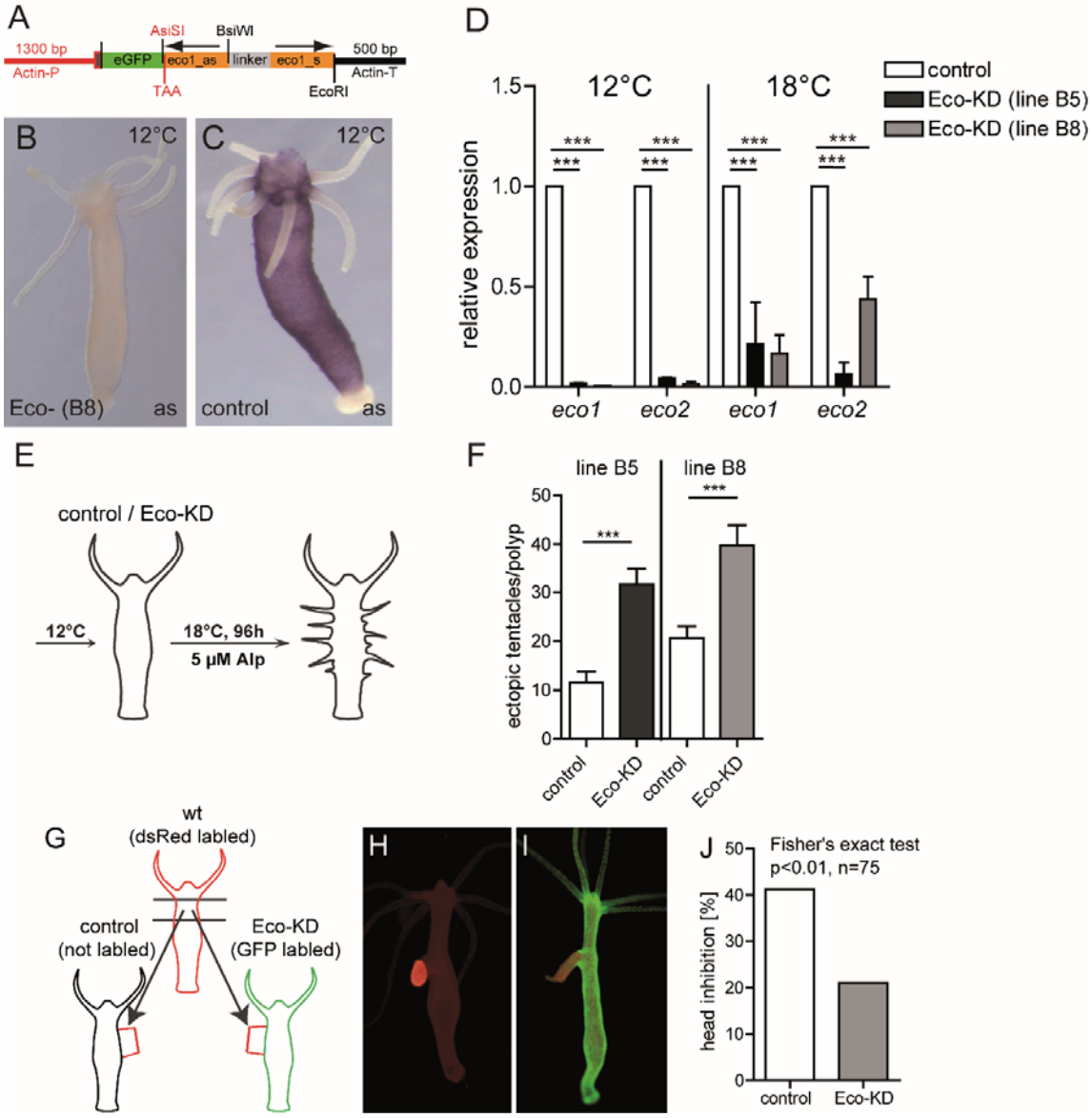

Figure 5: Knockdown of eco genes results in increased Wnt signaling. (A) Eco–hairpin construct for generation of transgenic *Hydra* (as, antisense; s, sense; TAA, stop codon; P, promoter; T, terminator). (B,C) Whole mount in situ hybridization of *eco1* expression in Eco-KD (B) and control (C) animals. (D) *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-F) Treating Eco-KD animals with ALP revealed an increased potential to form ectopic tentacles, indicating a higher Wnt signaling activity. (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 head inhibition potential, which is governed by the inhibition of the Wnt signaling. (H) Control (unlabeled) and (I) Eco-KD (GFP labeled) animals served as acceptor polyps for head tissues from wild type (dsRed labeled) animals to assess the head inhibition

potential under disturbed eco expression. (J) Eco-KD polyps showed a reduced head inhibition potential, indicating an impaired Wnt inhibition in these animals (n = 75, p=0.0085, Fisher's exact test).

To consolidate the notion of Eco peptides being an antagonist to Wnt-signaling, we performed transplantation experiments to measure the head inhibition potential of Eco-KD animals. Transplantation of Hydra tissue from apical body column regions into mid-body regions allows the formation of a secondary axis in the acceptor animal [38], depending on the position of excision from the donor and the site of transplantation in the acceptor [39]. Due to the head inhibition potential of the animal [39], the closer the pieces are transplanted to the head the lower the probability that a secondary axis is generated. Considering Eco peptides as antagonists to Wnt signaling, they might contribute to the head inhibition and thus affect axis stability in *Hydra.* Polyps with a reduced Eco level thus should be more prone to form secondary axis after transplantation compared to control animals (**Figure 5F**). We found, that the probability to form a secondary axis after transplantation is doubled in Eco-KD animals, compared to control animals, indicating a reduced head inhibition potential (**Figure 5G-I**). Together with the ALP experiment, these results demonstrated that Eco peptides antagonize the effects of Wnt-signaling.

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.

Discussion

Wnt-signaling: an evolutionary 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 [55]. In *Hydra* the Wnt pathway is involved in head formation [36], control of bud formation [56,57] and the differentiation of stem cells [58]. Increased Wnt-signaling causes the stem cells in *Hydra* to differentiate and to lose their potential to self-renew [58].

Here, we show that Eco peptides antagonizes the Wnt-signaling pathway (**Figure 6**). 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 promotor of *eco* genes, as both factors regulate the expression of both genes independently (**Figure 6**).

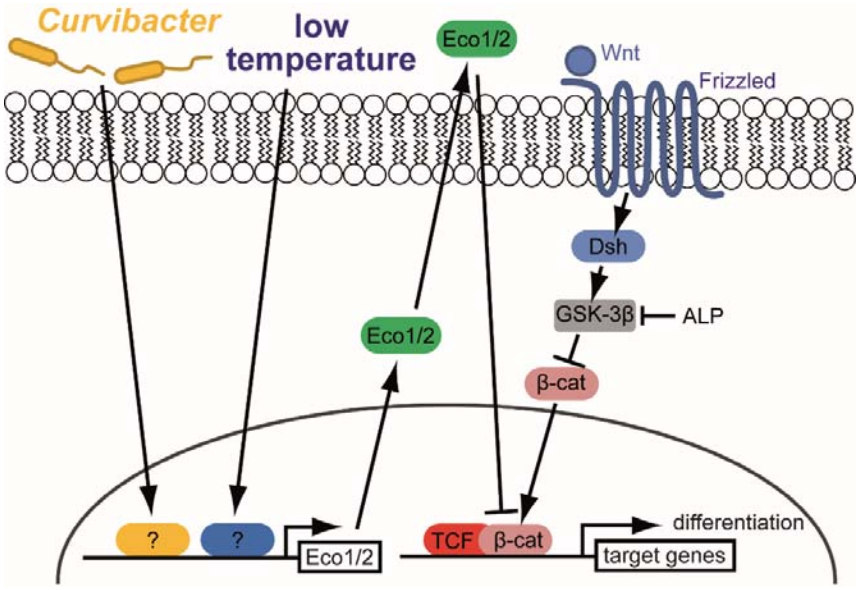

Figure 6: Eco peptides are environmentally triggered inhibitors of Wnt signaling. Microbial colonization and low temperature are independent inputs for Eco peptides and are able to induce its transcription. Eco peptides are synthesized as small peptides, packed into vesicles and secreted into the glycocalyx of the ectoderm. By an unknown mechanism Eco peptides are recognized and the Wnt signaling is inhibited. The inhibition takes place at the level of β-catenin stabilization or the expression of Wnt target genes as Eco expression were able to inhibit activation of the pathway at the GSK-3β level.

After activation of transcription, both peptides are translated and packed into vesicles. Most likely, the peptides are secreted to the apical side of the ectodermal epithelium into the

glycocalyx. Until now, it is unclear how Eco peptides antagonizes Wnt pathway, but we assume that they act downstream of β-catenin. We showed that low temperature is antagonizing the effect of overexpressed β-catenin. As β-catenin was mutated in a way that it is stabilized against APC dependent degradation [37], 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. Interestingly, the Eco peptides form opposing environmental-dependent gradients to the Wnt gradient, which is high in differentiated head tissue [36]. Interestingly, MacWilliams (1983) proposed a temperature dependent head inhibition mechanism, which can be explained by the interaction of Wnt and Eco ligands on the molecular level [39].

In our model we propose, that the ratio of recognized Wnt and Eco peptides by an individual cell determines the activation of Wnt-target genes. Thereby, the opposing gradients of Wnt and Eco determines the degree of differentiation in the tissue. We thus argue that the *eco* genes integrate environmental signals into the developmental program by counteracting with Wnt-signaling, and thereby promote stemness of the cells.

There are several studies in vertebrates showing the integration of environmental signals into the Wnt pathway. In zebrafish, the intestinal bacterium *Aeromonas veronii* enhances β-catenin stability resulting in higher cell proliferation in the intestine [59]. In human epithelial cells the CagA peptide produced by *Helicobacter pylori*, activates β-catenin, leading to transcriptional upregulation of genes implicated in cancer [60,61]. In addition, activation of the aryl hydrocarbon receptor by natural ligands, which are converted from dietary tryptophan and glycosinolates by intestinal microbes [62,63], results in the degradation of β-catenin [63] and the suppression of intestinal carcinogenesis. Together these results suggest that the Wnt pathway evolved early in animal evolution as a signaling hub integrating environmental signals, like bacterial signals and temperature, into developmental processes and stem cell behavior.

Orphan genes as circuit of environmental cues to host development

This study adds another example to taxonomic restricted or orphan genes as important factors for adaptations and stresses the importance of these fast evolving genes for the interaction with the environment [53,54].Orphan genes have been found to play important roles by recruiting new energy resources [64,65], and occupying new habitats [66]. Orphan genes often display functions in developmental programs [66,67], immunity [27,68] and mediate interaction with the environment [54,69,70]. Here we support this notion, by showing that the two eco paralogous interact with the environment in a way that the current conditions for temperature and microbiota association are sensed and signaled to the Wnt pathway. Thereby, the

developmental program is tuned by environmental factors via the regulation of the two orphan peptides Eco1 and Eco2.

Material and Methods

Animal culture. All experiments were carried out with either wild type or transgenic *Hydra vulgaris AEP (Hydra carnea* [71,72]) 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* 2-3 times the week.

Transgenic animals. The β-catenin over expression construct contained a truncated β-catenin (AA Δ1-138, containing the phosphorylation site important for Wnt signaling transduction) obtained from *Hydra magnipapillata* terminally fused to eGFP in a modified Hot-G Bluescript II SK + backbone (alias: pHyVec4, Steele, Genebank: DQ385853) [37]. The Eco1 hairpin constructs were cloned into a pGEM-T backbone obtained from *Hydra vulgaris* AEP (*Hydra carnea)* sequences (Compagen: HAEP_celera_v1_contig18166) containing base pair 3-303 with a 307 bp linker. dsRED and eGFP labelling constructs were codon usage optimized for *Hydra* and cloned into pGEM-T expression vectors. All constructs were driven by constitutively expressed *Hydra magnipapillata* actin promoters (~1.5 kb upstream the actin gene) and terminated by an actin terminator (~1 kb downstream of the gene). Stable transgenic animals were generated by microinjection of genetic constructs into 2-8 cell blastomere of *Hydra*. Transgenic animals hatched with a mosaic expression of eGFP, which served for selection of transgenic cells. The asexual mode of reproduction was used to select for transgenic cells and generate full transgenic animals on the on hand, while on the other hand non transgenic cells were selected to generate a non-transgenic line, which served as a control, containing the same genetic background.

Alsterpaullone experiment. Animals were fed one day prior experiment and treated with 5 µM Alterpaullone (ALP, *Sigma-Aldrich*) for 24 h to inhibit the glycogen synthase kinase 3 β (GSK3-β) and activate the Wnt signaling pathway. After treatment, the polyps were washed and incubated in HM before assessment of ectopic tentacle formation under the binocular 4 d after treatment.

Temperature treatment of β-catenin animals. β-catenin over expression animals were reared at either 18 °C or 12 °C and standard conditions prior 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, cDNA generation, qRT-PCR. Total RNA was isolated by dissolving 15-25 animals in 1 ml TRIzol (*Invitrogen*) by vortexing, after starvation for 2 days. After addition of 300 µL chloroform (p. a.), samples were centrifuged (12,000 g at 4 °C), the resulting aqueous phase was transferred in a clean tube, and 400 µl ethanol (99.9 %) was added. The solution was cleaned and desalted using the silica column protocol from Ambion PureLink RNA Mini Kit (*Thermo Scientific*), including an on column DNAse digestion step. 650 ng RNA was used to generate cDNA applying the protocol of the First Strand cDNA Synthesis Kit (*Fermentas*). Resulting cDNA was equilibrated within each experimental setup by semi-quantitative PCR using primer for the house keeping gene ELF1a. Equal amounts (according to PCR quantification) was used with the GoTaq qPCR Master Mix (*Promega*) in a 7300 real-time PCR system (*ABI*). For data analysis the simplified method after Pfaffl [73] was used, assuming a primer efficiency of 100 %.

In-situ hybridization. Whole open reading frame of *eco1* and *eco2* where cloned into pGEM-T (*Promega*). DIG-labeled probes where generated using T7/SP6 transcriptions start sites and the digoxigenin RNA Labeling Mix kit (*Roche*) after manufactures instructions. Hybridization was performed as described previously [74], in short: animals were relaxed with 2 % urethan for 2 min at 4 °C, fixed in 4 % PFA over night at 4 °C, washed three times á 10 min with PBT (PBS + 0.1 % Tween), and bleached in methanol (100 %) three times for minimum 30 min. Methanol was washed out with four decreasing concentrations of ethanol in PBT (100 %, 75 %, 50 %, 25 % v/v) followed by three times PBT washing steps, all 10 min or longer on a shaker. Samples were digested with 10 mg ml^{-1} Proteinase K in PBT for 20 min at room temperature and digestion was stopped by two subsequent washing steps with 4 mg ml^{-1} glycine in PBT, followed by three PBT washing steps each 10 min on a shaker. In order to acetylate positve charges in the samples and reduce background we treated samples twice with triethanolamine (0.1 M, pH 7.8) 5 min, once more with triethanolamine adding 2.5 µl $µm^{-1}$ acetic anhydrid for 5 min to add another 2.5 µl ml^{-1} acetic anhydrid for another 5 min. The samples where washed three times in PBT, refixed in 4 % PFA over night at 4 °C and washed another three times in PBT to remove fixative. Samples were treated twice with 2x SCC (0.3 M NaCl, 30 mM tri-sodium citrate, pH 7) for 10 min at room temperature and twice for 20 min at 70 °C, to inactivate endogenous phosphatases. To transfer the samples into hybridization solution (5x SSC + 50 % formamide (v/v), 0.02 % Ficoll (w/v), 0.02 % bovine serum albumin (w/v), 0.02 % polyvinylpyrolidone (w/v), 0.1 % Tween20 (v/v), 0.1 % CHAPS (w/v), 100 µg ml^{-1} Heparin), the medium was exchanged to prewarmed 50 % hybridization solution in 2x SSC for 10 min at 57 °C, followed by 10 min in hybridization solution at 57 °C and 2 h blocking in hybridization solution + 20 µl ml^{-1} tRNA at 57 °C. Around 20 ng $µl^{-1}$ In-situ probes were denatured for 10 min at 70 °C in 10x SSC +

50 % formamid. The denatured probe was added to the hybridization solution + tRNA to a final concentration of 2 ng μl^{-1} and incubated over night at 57 °C. Sense probes were used equally and served as negative control. To remove the probes and prepare antibody staining, the samples were washed in four steps of decreasing hybridization solution in 2x SSC (100 %, 75 %, 50 %, 25 %) for 10 min at 57 °C each. Afterwards, samples were washed twice in 2x SSC containing 0.1 % CHAPS for 30 min at 57 °C and twice in MAB-T (100 mM maleic acid, 150 mM NaCl, 0.1 % Tween20 (v/v), pH 7.5) for 10 min at room temperature. To block unspecific antibody binding sites, the samples were blocked with MAB-T + 0.1 % bovine serum albumin for 1 h at room temperature and with MAB-T + 0.1 % bovine serum albumin, 20 % heat inactivated sheep serum for 2 h at 4 °C. Anti-digoxigenin alkaline phosphate linked fab fragments (*Roche*) were used to detect probes by incubating the samples with a 1:2000 solution in MAB-T + 0.1 % bovine serum albumin, 20 % heat inactivated sheep serum over night at 4 °C. Excess antibody solution was removed by 8 washes with MAB-T for 15 min at room temperature. Sample staining was prepared by one NTMT (100 mM NaCl, 100 mM TRIS-HCl, 50 mM MgCl2, 0.1 % Tween20 (v/v), pH 9.5) wash and incubation with NTMT + 1 mM Levamisol for 5 min to inhibit unspecific endogenous alkaline phosphatases. Staining was performed by incubation of the samples in NTMT + 2 % NBT/BCIP in dark until a clear staining was obtained. The reaction was stopped with three washes of a*qua dest.* before dehydration in with three steps increasing ethanol concentrations (50 %, 70 %, 100 % in water) was performed and samples were embedded on glas slides in Euparal. Expression profiles of *eco* genes under the different treatments were analyzed under the microscope.

Immunohistochemistry. Immunostainging was performed using standard procedures [75]: animals were relaxed for in 2 % urethan for 2 min on ice and fixed in 4 % PFA over night at 4 °C. Fixative was removed with four washes in PBT (PBS + 0.1 % Tween20) for 15 min, cell membranes opened with PBS + 0.5 % TritonX100 for 30 min, and unspecific binding sites blocked with PBT + 1 % bovine serum albumin for 1h, eveything done at room temperature. The primary antibody was a polyclonal rabbit antibody raised against a 14 AA fragment (NSIKENMENFYPVE, AA 52-65, *GeneScript, USA*) of Eco1 and was incubated in PBT + 1 % bovine serum albumin, 10 µg/ml rabbit-α-Eco1-AB over night at 4 °C. The primary antibody was removed with four washes in PBT + 1 % bovine serum albumin for 15 min at room temperature. Secondary anitbody (goat-α-rabbit Alexa Flour 488 coupled, #A11034; LOT 1670152, *Life Technologies*) was incubated at 1 µg/ml in PBT + 1 % bovine serum albumin amd incubated for 2 h at room temperature, from this step on samples were kept in dark. Secondary antibody was removed by four washes in PBS + 0.5 % Tween20, 1 % bovine serum albumin for 15 min at room temperature. Actin cytoskeleton staining was performed

with rhodamine-phalloidin (#P1951, *Sigma*) in PBT for 1 h at room temperature and afterwards removed with four washes of PBS + 0.5 % Tween20, 1 % bovine serum albumin. To stain the nucleus we incubated the samples with TO-PRO3 iodide AlexaFluor633 (#T3605, LOT 23929W, *Invitrogen*) in PBS + 0.5 % Tween20, 1 % bovine serum albumin for 10 min at room temperature. Afterwards samples were transferred onto glas slides, embedded in Moviol and stored at 4 °C until analysis using a confocal laser scanning microscope (TCS SP1, *Leica*)

Transplantation experiments. Rings of tissue right beneath the tentacle ring was 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 2-3 h and fishing strings were removed. The induction of heads was assess 2-3 days post transplantation using the fluorescent markers and a binocular.

Author contributions

J. T., S. Franzenburg, T. C. G. B. and S. Fraune. designed the experiments; J. T. , M. K. , D. W.-O., and S. Franzenburg conducted experiments. J. T. , M. K. and S. Fraune performed data analysis. J. T. and S. Fraune wrote the paper, all authors corrected final version of the paper.

Acknowledgement

We greatly appreciate provision and microinjection of the *Hydra* embryos by Jörg Wittlieb. This work was funded by the German Research Foundation (DFG, CRC1182). T. C. G. B. gratefully appreciates support from the Canadian Institute for Advanced Research (CIFAR).

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