An evolutionarily-conserved Wnt3/β-catenin/Sp5 feedback loop restricts head organizer activity in Hydra

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Polyps of the cnidarian Hydra maintain their adult anatomy through two developmental organizers, the head organizer located apically and the foot organizer basally. The head organizer is made of two antagonistic cross-reacting components, an activator, driving apical differentiation and an inhibitor, preventing ectopic head formation. Here we characterize the head inhibitor by comparing planarian genes down-regulated when β-catenin is silenced to Hydra genes displaying a graded apical-to-basal expression and an up-regulation during head regeneration. We identify Sp5 as a transcription factor that fulfills the head inhibitor properties: leading to a robust multiheaded phenotype when knocked-down in Hydra, acting as a transcriptional repressor of Wnt3 and positively regulated by Wnt/β-catenin signaling. Hydra and zebrafish Sp5 repress Wnt3 promoter activity while Hydra Sp5 also activates its own expression, likely via β-catenin/TCF interaction. This work identifies Sp5 as a potent feedback loop inhibitor of Wnt/β-catenin signaling, a function conserved across eumetazoan evolution.
The freshwater *Hydra* polyp, which belongs to Cnidaria, a sister group to Bilateria, has the remarkable talent to regenerate any lost body parts, including a fully functional head. *Hydra*, which is made of two cell layers, external named epidermis and internal named gastrodermis, shows a polarized tubular anatomy with a head at the apical/oral pole and a foot at the basal/aboral one, both extremities being enriched in nerve cells (Fig. 1a). Remarkably, the cnidian oral pole has been proposed to correspond to the posterior end of bilaterians. Head regeneration relies on the rapid transformation of a piece of somatic adult tissue, the amputated gastric tube, into a tissue with developmental properties named head organizer, which directs the patterning of the regenerating tissue (reviewed in [2–4](#)). In *Hydra* regenerating its head, the organizer gets established within 10 to 12 h after mid-gastric bisection, restricted to the head-regenerating tip within the first 24 h, remaining stable until the new head is formed and subsequently persisting as a homeostatic head organizer.

The *Hydra* model also helped understand the dual structure of organizers. By comparing the efficiency of apical grafts to induce ectopic axis on intact or decapitated hosts, Rand et al. showed that the *Hydra* head organizer exerts two opposite activities, one activator that promotes apical differentiation, and another inhibitor that prevents the formation of supernumerary or ectopic heads. In *Hydra* the inhibitory activity is graded along the body axis, maximal at the apical pole, and tightly modulated during head regeneration, rapidly decaying after amputation and slowly recovering. Gierer and Meinhardt used the results obtained from a series of transplantation experiments to propose a general mathematical model of morphogenesis. Their model revisits the Turing model based on the reaction-diffusion model, where two antagonists, form a minimal regulatory loop that suffices for de novo pattern formation. Among these candidates, we found *Wnt3* and *Wnt5*, known as positive regulators of morphogenetic processes, upregulated in both head- and foot-regenerating tips but its expression is only sustained in head-regenerating ones (Fig. 1c, d). Among these candidates, we found *Wnt3* and *Wnt5*, known as positive regulators of morphogenetic processes, upregulated within the first day of head regeneration, (4) inhibit Wnt/β-catenin signaling, (5) prevent head formation (Fig. 1b). To select β-catenin target genes, we used a dataset of 440 genes downregulated in planarians silenced for β-catenin to retrieve 124 *Hydra* cognate genes (Supplementary Data 1). We analyzed their spatial and temporal RNA-seq expression profiles and found 5/124 genes predominantly expressed in the head and 3/5 upregulated in head-regenerating tips at least 1.5 fold after 24 h of regeneration (Fig. 1c, d). Among these candidates, we found *Wnt3* and *Wnt5*, known as positive regulators of morphogenetic processes, and *Sp5*, previously identified as a Wnt/β-catenin target gene in vertebrates, thus a putative HI candidate (Fig. 1e). *Hydra* Sp5 (HySp5) encodes a Sp/KIF-class transcription factor whose sequence clusters with the bilaterian Sp5 ones in phylogenetic analyses (Supplementary Figs. 1–3).

Whole mount in situ hybridization confirmed the RNA-seq pattern in intact *Hydra*, predominantly expressed in the head although absent from the apical tip where *Wnt3* expression is maximal (Fig. 1f, g). After mid-gastric bisection, Sp5 is rapidly upregulated in both head- and foot-regenerating tips but its expression is only sustained in head-regenerating ones (Fig. 1g, Supplementary Fig. 4) supporting the idea that Sp5 is involved in head but not foot regeneration. We also performed a RNA-seq analysis of the cell-type expression and found that both Sp5 and Wnt3 are predominantly expressed in the gastrodermal epithelial stem cells (gESCs), a cell type associated with morphogenetic processes (Supplementary Fig. 5).

### Results

**Identification of putative *Hydra* head inhibitors.** To identify inhibitors of apical patterning that regulate the activity of the head organizer in both homeostatic and regenerative conditions, we established five criteria to be fulfilled by head inhibitor (HI) gene(s): (1) be controlled by Wnt/β-catenin signaling, (2) display an apical-to-basal graded activity, (3) be upregulated within the first day of head regeneration, (4) inhibit Wnt/β-catenin signaling, (5) prevent head formation (Fig. 1b). To select β-catenin target genes, we used a dataset of 440 genes downregulated in planarians silenced for β-catenin to retrieve 124 *Hydra* cognate genes (Supplementary Data 1). We analyzed their spatial and temporal RNA-seq expression profiles and found 5/124 genes predominantly expressed in the head and 3/5 upregulated in head-regenerating tips at least 1.5 fold after 24 h of regeneration (Fig. 1c, d). Among these candidates, we found *Wnt3* and *Wnt5*, known as positive regulators of morphogenetic processes, and *Sp5*, previously identified as a Wnt/β-catenin target gene in vertebrates, thus a putative HI candidate (Fig. 1e). *Hydra* Sp5 (HySp5) encodes a Sp/KIF-class transcription factor whose sequence clusters with the bilaterian Sp5 ones in phylogenetic analyses (Supplementary Figs. 1–3).

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**Hydra Sp5 a robust head inhibitory component.** Next, we silenced Sp5 by electroporating siRNAs in intact animals and observed that within two days following the third electroporation (RNAi3), Sp5(RNAi) animals develop ectopic axes, initially from the budding zone, few days later from the upper body column (Fig. 2a, Supplementary Fig. 6). These ectopic axes differentiate multiple heads when located in the basal half but not from the upper half. Both ectopic axes and ectopic heads express the apical markers *Wnt3*, *Bra1* and *Tsp1*, and the gland cell marker *Kazal1* in the gastric tissue (Fig. 2b). When single-headed animals silenced for Sp5 are bisected after RNAi2, they all regenerate multiple heads that express *Wnt3* at the tip (Fig. 2c, Supplementary Fig. 7). This multiheaded phenotype is
robust, emerging quite synchronously in 50% uncut animals one day after RNAi2, in 100% two days after RNAi3 (Fig. 2d, Supplementary Fig. 6a–c). Furthermore, these ectopic heads express the neuropeptide RF-amide and are able to catch and ingest live *Artemia*, indicating that each ectopic head is functional (Fig. 2e, Supplementary Fig. 6d, Supplementary Movies 1–4). These results indicate that Sp5 acts as a strong inhibitor of head formation in *Hydra*.

**Sp5 antagonizes Wnt/β-catenin signaling in *Hydra***. Next, we tested whether the multiheaded phenotype corresponds to a de-repression of Wnt3. To do this, we first tested whether the phenotype occurs when the Wnt/β-catenin pathway is inactive and thus knocked-down Sp5 together with β-catenin (Fig. 3a). Silencing β-catenin on its own delays head regeneration (Supplementary Fig. 8) and causes the formation of ectopic bumps in intact animals (Fig. 3a, Supplementary Fig. 9). While knocking
down Sp5 causes the formation of multiple heads, the simultaneous knockdown of Sp5 and β-catenin prevents the occurrence of the multiheaded phenotype (Fig. 3a, Supplementary Fig. 9), suggesting that an increase in Wnt/β-catenin signaling activity is necessary to trigger multiple head formation when Sp5 is knocked down.

To further demonstrate that Sp5 represses Wnt/β-catenin signaling via Wnt3 repression, we knocked-down Sp5 in combination with Alsterpaullone (ALP), a drug that activates the Wnt/β-catenin pathway by antagonizing GSK3β. As anticipated this combination led to a significant increase in ectopic tentacle formation, while knocking down β-catenin provides the opposite effect (Fig. 3b, Supplementary Fig. 10a).

In these Sp5(RNAi) animals, we could also detect an increase in Wnt3 expression along the body column, indicating that Sp5 does repress Wnt3 expression (Fig. 3c, Supplementary Fig. 10b).

We also performed reaggregation experiments with cells coming from ALP-treated animals knocked-down either for Sp5 or for β-catenin. In standard conditions of reaggregation, several head spots form, each of them containing 5 tentacles along the body column (Supplementary Fig. 12a). In such transgenic animals expressing a Wnt3 promoter, we could identify regions with a number of Wnt3 expressing spots increased by two-fold (Fig. 3d, e, Supplementary Fig. 11). In contrast, when β-catenin is knocked-down, the reaggregation process proceeds slower with aggregates exhibiting only few tentacles at day-4, with a number of Wnt3-expressing clusters similar to that observed in scramble(RNAi) control animals (Fig. 3d). These results confirm that Sp5 directly or indirectly represses Wnt3 expression.

To test whether Sp5 can directly repress the Wnt3 promoter, we produced a transgenic strain expressing the HyWnt3–2149: GFP-HyAct:dsRed construct where 2′149 bp of the Hydra Wnt3 promoter drives GFP expression and the Hydra Actin promoter drives dsRed expression. We noted distinct levels of Wnt3-driven GFP fluorescence in control transgenic animals, maximal at the apex, intermediate in the adjacent region above the tentacle ring, and null at the level of the tentacle ring and along the body column (Supplementary Fig. 12a). In these transgenic animals knocked-down for Sp5, we did not record any body-wide GFP fluorescence but rather the appearance of patches of GFP+ cells at the tip of the ectopic axes (Supplementary Fig. 12b). We could confirm this patch Wnt3 activation along the body column of Sp5(RNAi) animals by performing a detailed kinetic analysis of Wnt3 expression (Supplementary Fig. 12c–d). This Wnt3 ectopic expression pattern suggests that Sp5 is silenced in restricted regions along the body column where Wnt3 is de-repressed and enhances its own expression via β-catenin signaling as previously recorded.

Sp5 represses the Hydra and zebrafish Wnt3 promoter. To further investigate the repressing activity of HySp5 on the HyWnt3 promoter, we performed luciferase reporter assays in human HEK293T cells (Fig. 4a–c). As the HyWnt3–2149:Luc construct shows a very low basal activity, we co-expressed a constitutively active form of β-Catenin (CMV:huβ-Cat) that enhances by ~10-fold the luciferase activity (Fig. 4b). In such conditions, the co-expression of HySp5 significantly reduces the activity of the HyWnt3 promoter (Fig. 4b). This effect was not observed when a partial version of HySp5 lacking the DNA-binding domain was used, indicating that the repressive effect of HySp5 is DNA-binding dependent (Fig. 4b). Two adjacent cis-regulatory modules were previously identified in the HyWnt3 promoter, a 599 bp-long activator that contains three clustered TCF binding sites and a 386 bp-long repressor sequence, located immediately downstream (Fig. 4a, Supplementary Fig. 13a). This repressor module, highly conserved across Hydra species (Fig. 4a), is necessary for the Sp5-mediated Wnt3 repression, as the repression is no longer observed when this element is removed (Fig. 4b). Among the four constructs that harbor limited deletions within the Wnt3 repressor element, the construct containing both the -386/-286 and the -95/-1 sequences is the only one repressed by Sp5 (Fig. 4c), suggesting that the Sp5-dependent Wnt3 repression requires the cooperative activity of these two elements.

To test whether Sp5 also represses Wnt3 transcription in vertebrates we tested the 4 kb promoter region of the zebrafish Wnt3 locus in reporter assays where the zebrafish paralogs ZfSp5a and ZfSp5l1 are expressed (Fig. 4d, e). As for the HyWnt3–2149 construct, the transcriptional activity of the ZfWnt3–3997 construct was strongly enhanced by huβ-Cat, but repressed upon co-expression of ZfSp5a or ZfSp5l1 (Fig. 4e). The repressor activity of ZfSp5a was abolished when the DNA-binding domain was deleted. Although the zebrafish Wnt3 promoter does not share obvious sequence homologies with that of the HyWnt3 promoter, we could identify regions evolutionarily-conserved across different teleost lineages as well as TCF binding sites (Fig. 4d, Supplementary Fig. 13b). ChIP-qPCR experiments performed in transfected HEK293T identified two evolutionarily-conserved elements within the ZfWnt3 promoter directly bound by ZfSp5a (Fig. 4f).
HySp5 promoter, a region that is evolutionarily-conserved across Hydra species and contains five putative TCF binding sites (Fig. 5b, Supplementary Fig. 13c). We evidenced its responsiveness to Wnt/β-catenin signaling, as we recorded a significant upregulation of the activity of the HySp5–2992:Luc reporter construct when the human WNT3, LRP6 or huβ-catenin proteins were co-expressed (Fig. 5c). In addition, we found that HySp5 can bind its own promoter as in ChIP-qPCR experiments Sp5 binding was significantly enriched in two neighboring regions located immediately upstream of the Sp5 Transcriptional Start Site (TSS) (Fig. 5d). Furthermore, co-expression of HySp5–2992:Luc and HySp5, alone or in combination with huβ-catenin, resulted in a strong increase in luciferase activity (Fig. 5e). In mouse ESCs, Sp5 interacts with β-catenin and Tcf-Lef1 to regulate gene expression\(^3\). As anticipated, we found in a ChIP-seq analysis the mouse Sp5 and β-catenin proteins enriched in the same region of the Sp5 promoter (Supplementary Fig. 15a), suggesting a possible cooperation to regulate Sp5 transcription. We performed co-immunoprecipitation experiments with HEK293T cells co-transfected with HySp5 and huβ-catenin or huTCF1 and observed an interaction between HySp5 and these factors (Fig. 5f, Supplementary Fig. 15b–c). These results indicate that HySp5, similarly to its mammalian cognates, can act as an activator or a repressor of transcription and that Hydra and vertebrate Sp5 can interact with β-catenin or TCF1.

Sp5 DNA-binding properties are evolutionarily-conserved. To further compare the transcriptional activities of HySp5 and ZfSp5a, we expressed HySp5 or ZfSp5a in HEK293T cells and analyzed the genomic occupancies and the transcriptional changes induced by their overexpression (Fig. 6a). ChIP-seq analysis revealed that HySp5 binds a much smaller fraction of sequences than ZfSp5a (Fig. 6b), while the number of genes bound by HySp5 and ZfSp5a is not so different, 13'251 vs. 18'619, 99% of the HySp5 bound genes are also ZfSp5a targets (Fig. 6c). Interestingly, HySp5 and ZfSp5a differ in the spatial distribution of their target sequences: the majority of HySp5 bound elements localize within the 5 kb proximal region of the assigned genes, while ZfSp5a proportionally binds more frequently elements...
located in upstream sequences, above 10 kb from the TSS (Fig. 6d, e). This suggests that vertebrate Sp5 more readily recognizes sequences enriched in long-range regulatory elements, which are not recognized by the HySp5 protein.

Motif enrichment analysis of the HySp5 and ZfSp5a bound elements revealed that the two orthologs recognize both similar and divergent consensus binding sites (Fig. 6f). In both cases, the most enriched motif resembled the general SP/KLF consensus sequence (GGGxGGG/A). We then used the enriched motifs to identify putative HySp5/ZfSp5a binding sites in the regulatory regions of HyWnt3, ZfWnt3 and HySp5. We could identify putative HySp5 binding sites in the two regions of the HyWnt3 repressor required to inhibit transcription (Supplementary Figs. 13). Similarly, we also found evolutionarily-conserved Sp5 binding sites in the regions of ZfWnt3 and HySp5 enriched in the ChIP-qPCR analysis, supporting the idea that Hydra and

**Fig. 3** HySp5 antagonizes Wnt/β-catenin signaling in Hydra. a Double knockdown of Sp5 and β-catenin. Animals were pictured live on day-8 in two independent experiments. RNAi: siRNA electroporation day. Red arrows: ectopic heads; white arrows: bumps. b Knockdown of Sp5 and β-catenin in ALP-treated animals. Upper panel: Morphological changes observed in animals fixed at day-7. Lower panel: Quantification of ectopic tentacles formed at day-7. Each dot represents the number of ectopic tentacles in one animal (two biologically independent experiments). c Intact Hydra exposed to siRNAs and ALP as indicated and detected for Wnt3 by WISH (upper panel) and qPCR (lower panel). Blue arrows: RNA extraction days; black arrows: local increase in Wnt3; black arrowhead: diffuse increase in Wnt3 expression. Each data point represents one biologically independent experiment. d Effect of Sp5 knockdown on head patterning during the process of reaggregation initiated on day-4 (black arrow). Aggregates were pictured live (upper row), then fixed to detect Wnt3 expression (lower row) in two independent experiments. e Quantification of Wnt3 expressing clusters. Each dot represents the number of Wnt3+ clusters in one aggregate. Statistical p values: *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001 (unpaired t test). Scale bars in panels a–d: 200 μm. Error bars indicate SD.
zebrafish Sp5 directly regulate the transcriptional activity of these promoters. Despite the similarity in the main consensus sites bound by HySp5 and ZfSp5a, we also identified motifs differentially enriched among the elements bound by these two orthologs (Fig. 6f). Interestingly, ZfSp5a binds elements that display an over-representation of Tbx/Sox motifs, which were not identified in the pool of HySp5 bound sequences (Fig. 6f). Members of the Tbx and Sox families are known to interact with Sp5 and β-catenin respectively, suggesting that they could also form transcriptional complexes with Sp5. Thus, the enrichment in Tbx/Sox consensus sequences suggests that vertebrate Sp5 but not Hydra Sp5 may regulate gene expression in complexes involving these transcription factors.

To further validate that HySp5 has similar DNA-binding properties than its vertebrate orthologs, we inspected the HySp5 genomic coverage in the proximities of genes identified by ChIP-qPCR assays (Fig. 6f). We observed a higher number of peaks in the HySp5 dataset compared to the ZfSp5a dataset, suggesting a higher binding affinity of HySp5 to DNA. Additionally, we performed luciferase assays to measure the activity of the Wnt3 promoter when deleted of different portions of the repressor. Note that the repressive effect of HySp5 is only observed with the HyWnt3-2149 and HyWnt3-ΔRep-D3 constructs. (d) Phylogenetic footprinting comparing the 4 kb genomic region encompassing the zebrafish Wnt3 promoter with the corresponding genomic regions of three teleost fish species. Pink peaks in the Vista alignment plot represent evolutionarily-conserved modules (at least 70% base-pair identity over 100 bp sliding window).

**Fig. 4** Hydra Sp5 and zebrafish Sp5 repress the Wnt3 promoter activity. **a** Map of the HyWnt3 promoter and phylogenetic footprinting plot comparing the 2 kb genomic region encompassing the *H. magnipapillata* (*Hm-105*) Wnt3 promoter with the corresponding regions in the *H. oligactis* and *H. viridissima* genomes. Green and magenta bars indicate the activator and repressor regions identified by ChIP-qPCR assays performed with cells expressing ZfSp5a or ZfSp5a-ΔDBD. Note that members of the Tbx and Sox families are known to interact with Sp5 and β-catenin respectively, suggesting that they could also form transcriptional complexes with Sp5. Thus, the enrichment in Tbx/Sox consensus sequences suggests that vertebrate Sp5 but not Hydra Sp5 may regulate gene expression in complexes involving these transcription factors.
Wnt/β-catenin targets in mouse and human ESCs. Comparative binding profiles of HySp5 and mSp5 were observed for the Axin2, β-catenin, Wnt3a, Axin2, VdII, and Lrg5 loci in human and mouse cells, while quite different at the Nanog and Plk4 loci, the latter likely due to cell-type or species specific differences (Supplementary Fig. 16a). We also found a strong enrichment of HySp5 and ZfSp5a binding in the WNT3 intronic sequences, in the promoter and intronic sequences of the neighboring WNT9B locus and in the upstream and intronic sequences of SP5 (Supplementary Fig. 16b). The GO term enrichment analysis actually identified the Wnt pathway as the most enriched category (Supplementary Fig. 16c, Supplementary Data 2).

All together, these results point to similar DNA-binding capacities between HySp5 and ZfSp5a even though the latter recognizes a larger set of sequences, often located at mid-long distances upstream from the TSS, possibly acting in combination with Sox and/or Tbx proteins.

Conserved and divergent transcriptional functions of Sp5. To assess the transcriptional activity of HySp5 and ZfSp5a, we measured by qRNA-seq the transcriptional changes induced by the overexpression of HySp5 and ZfSp5a in HEK293T cells co-expressing or not the huΔβ-Cat construct (Fig. 6a). As controls we used HEK293T cells transfected with a mock plasmid, the huΔβ-Cat construct alone or the mutated HySp5ΔDBD and ZfSp5aΔDBD constructs. Principal component analysis (PCA) showed that HySp5 and ZfSp5a transfected samples, either alone or in combination with huΔβ-Cat, segregated together, widely separated from the control or HySp5ΔDBD/ZfSp5aΔDBD values (Fig. 6g). This suggests that HySp5 and ZfSp5a elicit overall similar transcriptional responses. Instead, the values obtained from huΔβ-Cat transfected cells grouped together with the values from mock-transfected samples, while the values corresponding to cells co-expressing huΔβ-Cat with HySp5 or ZfSp5a do not substantially differ from those overexpressing HySp5 or ZfSp5a alone (Fig. 6g, Supplementary Data 2). These results imply that HEK293T cells do not respond to HuΔβ-Cat overexpression, in agreement with previous reports showing that although HEK293T cells respond to Wnt signalling stimulation by translocating β-catenin to the nucleus, they display limited transcriptional responses of their endogenous Wnt target genes.

Next, we analyzed the genes whose expression is modulated upon HySp5 or ZfSp5a overexpression but remains unaffected when their respective DNA-binding domain is deleted (Fig. 6h, Supplementary Data 2). We focused our analysis on the modulated genes that were associated to HySp5- or ZfSp5a-bound elements in ChIP-seq analysis, suggesting that these genes are directly activated or directly repressed targets. We identified downregulated genes, 153 upon HySp5 expression, 113 by ZfSp5a, and 83 by both (Fig. 6i, Supplementary Fig. 17, Supplementary Data 3). This demonstrates that the cnidarian and vertebrate Sp5 proteins have a similar repressive capacity. We also identified 137 and 23 genes upregulated upon ZfSp5a and HySp5 overexpression, respectively. Of these, only 5 are activated by both Sp5 orthologs (Fig. 6i, Supplementary Fig. 17, Supplementary Data 3), indicating that the activator function of the cnidarian and vertebrate Sp5 transcription factors diverged.
RNA-seq data in this study. Putative target genes were identified based on the identification of Sp5 bound elements using ChIPenrich. The consensus motifs enriched in Sp5 bound elements were identified using the MEME ChIP suite tool. Differential expression analysis was performed on RNA-seq samples to identify up- and downregulated genes. Those associated to an Sp5 bound element were considered as direct Sp5 up- or downregulated targets.

**Fig. 6** Hydra Sp5 acts as a transcriptional activator and repressor. a Schematic representation of the workflow used for the analysis of the ChIP-seq and RNA-seq data in this study. Putative target genes were identified based on the identification of Sp5 bound elements using ChIPenrich. The consensus motifs enriched in Sp5 bound elements were identified using the MEME ChIP suite tool. Differential expression analysis was performed on RNA-seq samples to identify up- and downregulated genes. Those associated to an Sp5 bound element were considered as direct Sp5 up- or downregulated targets. b Bar graph representing the genomic coverage of HySp5 and ZfSp5a genome wide or in the promoters of Ensembl genes (defined as the 5 kb upstream of the gene TSS). Only autosomal chromosomes were used for this study. ZfSp5a coverages are considerably higher than those of HySp5. However, within gene promoters this difference is proportionally lower. c Venn diagram representing the number of genes assigned to HySp5 or ZfSp5a bound elements. Note the considerable overlap between HySp5 and ZfSp5a data sets. d Bar plot representing the percentage of Sp5 bound elements at different distances from the assigned gene TSS for HySp5 (blue) or ZfSp5a (green). e Frequency distribution of the number of peaks associated to each gene and located in the promoter region (left), 10–100 kb upstream of the gene TSS (middle) or at long genomic distances (>100 kb) from the gene body. f Enriched transcription factor consensus matrix identified in HySp5 and ZfSp5a bound elements. g Principal component analysis showing the segregation of RNA-seq samples across the two main principal components. h Heat map plots showing the z score value of genes significantly up- or downregulated (based on Wald test p < 0.05) in HySp5 or ZfSp5a transfected HEK293T cells compared to their respective control conditions (HySp5+ ΔDBD or ZfSp5a+ΔDBD). i Venn diagrams showing the number of HySp5 and ZfSp5a direct transcriptional targets (see description in a) significantly up- or downregulated.
during evolution. This is surprising, since both HySp5 and mammalian orthologs can interact with β-catenin (Fig. 5) to promote target gene activation. As the HEK293T cells are largely insensitive to huΔp-Cat overexpression (Fig. 6g), the observed upregulation of HySp5 and ZfSp5a direct targets relies on mechanisms largely independent of β-catenin signaling. By contrast, the overexpression of HySp5 and ZfSp5a in zebrafish embryos leads to similar developmental alterations, which resemble those produced by the over-activation of Wnt/β-catenin signaling (Supplementary Fig. 18, Supplementary Data 4).

Discussion

Studies performed in developing vertebrates show that Sp5 is a target of Wnt/β-catenin signaling as recorded in zebrafish27,28, mice29, Xenopus30, as well as in self-renewing mouse and human ESCs31,32. In line with these results, we show that in Hydra, Sp5 is positively regulated by Wnt/β-catenin signaling as evidenced by its upregulation when Wnt/β-catenin signaling is pharmacologically enhanced. These results illustrate the deep conservation of the Wnt/β-catenin-dependent regulation of Sp5 across eumetazoa. Wnt5, another candidate identified in the screen might also play a role in head inhibition, as a putative inhibitor of the canonical Wnt pathway33,34 and a possible HySp5 target gene. By contrast, secreted Wnt antagonists such as Dickkopf (Dkk)35 or Notum36, both expressed in Hydra, were not identified in this screen.

Wnt3 and Sp5 upregulations in head-regenerating tips are consistent with a rapid head organizer formation after bisection. Sp5 is re-expressed early during head regeneration, although as expected, later than Wnt3. This temporal parameter is indeed essential for the establishment of a de novo head organizer as demonstrated by transplantation experiments that accurately measured the successive re-activation of the two head organizer components, with head activation restored within 12 hpa and head inhibition coming back later, detectable at 24 hpa.13 Here we used the qRNA-seq data to compare the respective regulations of Wnt3 and Sp5 in regenerating tips after decapitation or mid-gastric bisection. While Wnt3 is rapidly upregulated to reach a plateau value at 4 hpa, Sp5 shows an initial drop in expression within the first two hours following bisection, then an upregulation and a peak of expression detected at 8 hpa, four hours after that measured for Wnt3. If one assumes that the reestablishment of active Wnt3 and Sp5 proteins follows similar kinetics, then this four hour time window corresponds to a period when Wnt3/β-catenin signaling is active but Sp5 still inactive as Wnt3 repressor, leaving sufficient time to instruct tissues to form a head.

A recent observation suggested that human Sp5 can directly repress the WNT3 promoter in human ESC37. Here we demonstrate that indeed Sp5 from Hydra and zebrafish inhibit Wnt/β-catenin signaling by repressing the activity of the WNT3 promoter. Both the RNA-seq and the ChIP-seq data presented here confirm this view, by showing firstly that HySp5 and ZfSp5a when overexpressed in HEK293T cells repress largely overlapping sets of genes and secondly that both Hydra and zebrafish Sp5 preferentially bind genes of the Wnt/β-catenin signaling pathway, as observed in the promoter and intronic regions of the human WNT3 and WNT9B genes. The studies performed in HEK293T cells also highlighted the fact that HySp5 and ZfSp5a, as transcriptional repressors, likely bind to regulatory elements located in the proximity of the TSS of their target genes. All together, these results highlight the similarity between the repressor effect of cnidarian and vertebrate Sp5 transcription factors, which predominantly affects genes of the Wnt/β-catenin signaling pathway but is not restricted to it. It is thus tempting to speculate that the Sp5-dependent inhibition of Wnt/β-catenin signaling originated early in metazoan evolution and was maintained across eumetazoa. By contrast, the properties of HySp5 and ZfSp5a as transcriptional activators appear quite different: both can promote gene activation through β-catenin interaction, but they largely differ in their capacity to activate target genes in a β-catenin-independent mode. Therefore, we speculate that Sp5 possibly evolved the capacity to interact with partners not previously identified such as Tbx or Sox, and/or acquired the capacity to bind consensus motifs such as those enriched in the vertebrate long-range enhancers, after Cnidaria divergence.

Consistent with its Wnt3 repressor function, HySp5 silencing triggers in a highly robust way the ectopic formation of clusters of Wnt3-expressing cells, followed by the formation of multiple heads along the body column of intact animals, in head-regenerating regions and in reaggregates (Fig. 7). This phenotype is different from the ones obtained with pharmacological treatments, either with the GSK3-β inhibitor ALP22,23,33 or recombinant Wnt3 that directly enhances β-catenin signaling18,47, where ectopic tentacles form first, and heads appear several days later. In intact animals, the knockdown of HySp5 leads to the direct and rapid formation of fully functional ectopic heads, preferentially in the budding zone, a region that is developmentally competent in adult animals where the expression of both Wnt3 and β-catenin is quite dynamically regulated17,18. By increasing the number of dsRNA electroporations, we noted the formation of ectopic heads in the apical half of the body column, even though the development of these heads remained incomplete. Nevertheless, we never observed super-numerary heads at the apex of homeostatic HySp5(RNAi) animals, likely reflecting the difficulty to obtain a significant silencing in the apical region where Sp5 expression is high. In the peduncle and basal part of the animal, ectopic head formation upon HySp5(RNAi) does not occur either, most likely as the physiological activity of Wnt3/β-catenin signaling is too low in this region to elicit ectopic head formation when Sp5 is silenced. In head-regenerating animals or reaggregates, the Sp5(RNAi) phenotype is readily observed as, similarly to the budding zone, the expression of Wnt3, β-catenin and Sp5 is quite dynamically regulated.

To further investigate these dynamic modulations, we designed strategies to modulate the Sp5(RNAi) phenotype. We first noticed that when β-catenin is silenced, the Sp5(RNAi) phenotype is greatly reduced, indicating that an active Wnt3/β-catenin signaling is necessarily required for ectopic head formation. We also measured the spatial spreading of the ALP-induced phenotype when Sp5 is knocked-down, with ectopic Wnt3 expression and ectopic tentacle formation all along the body column. This last result indicates that the constitutive activation of Wnt3/β-catenin signaling by ALP is significantly enhanced upon Sp5 silencing. These modulations of the Sp5(RNAi) phenotype in response to β-catenin(RNAi) or the ALP-induced phenotype in response to Sp5 (RNAi) again confirm the intimate dynamic cross-talk that takes place between Sp5 regulation, Sp5 activity and the Wnt3/β-catenin signaling activity.

The observed Sp5(RNAi) phenotypic modulations indicate that Sp5 silencing cannot be easily maintained stable along the mid-gastric region, namely because its regulation is quite dynamic in response to the level of Wnt3/β-catenin signaling. Therefore, we interpret the homeostatic HySp5(RNAi) phenotype in the budding region as the consequence of the transient downregulation of HySp5 activity in tissues that have the highest potential for setting up an organizer as evidenced by the transient upregulation of β-catenin in the budding zone17. As an evidence of this dynamic cross-talk, we noticed that a transient drop in HySp5 expression suffices to rapidly induce a de-repression of Wnt3 expression, which leads to an upregulation of β-catenin activity, and in turn
to Wnt3 upregulation followed by that of Sp5 (Fig. 7). The oscillatory nature of HySp5 and β-catenin expression in regions competent for head organizer formation suggests a bistability state relying on an auto-regulatory loop involving two transcription factors. This bistability as a prerequisite to head organizer induction and/or head organizer maintenance remains to be explored.

This study identifies the transcription factor Sp5 as a key inhibitory component of the Hydra head organizer. Indeed Sp5 fulfills the five criteria we initially fixed, derived from the predicted properties of the head inhibitor and from the previous identification of Wnt/β-catenin signaling as the head activator. Sp5 globally fits the Turing/Gierer-Meinhardt model as HySp5 expression is controlled by Wnt3/β-catenin signaling, predominantly expressed in the head, reactivated during head regeneration, while HySp5, as a Wnt3 repressor, represses ectopic head formation (Fig. 7). However, several features diverge from the expected properties of the head inhibitor predicted by the Gierer-Meinhardt model.

Firstly, we noted the lack of Sp5 expression at the very apical tip of the hypostome in intact animals, the region where Wnt3 expression, and most likely Wnt3 activity, is maximal. Two distinct cis-regulatory elements in the Wnt3 promoter were previously identified, an activator and a repressor element, the latter restricting Wnt3 expression to the distal tip of the head. The Sp5 pattern is thus consistent with the prediction that the inhibitor should be absent or unable to repress Wnt3 in this area. As Sp5 appears as a direct target of Wnt3/β-catenin signaling (see below), an additional negative regulation has to take place in this most apical area, to prevent Sp5 expression. This local regulation remains to be identified.

Secondly, this study supports a scenario where Wnt3 acts as a short-range activator to sustain its own activity in the head organizer, while Sp5 prevents the expression of Wnt3 and possibly other Wnt genes in non-apical tissues. The Gierer-Meinhardt model, proposed at a time when the concept of transcription factor was still unknown, predicts that the head inhibitor is a diffusible substance, acting non-cell autonomously across the tissue layers. As a transcription factor, HySp5 is suspected to act cell-autonomously and thus not diffusible. However, some transcription factors can be secreted, as reported for the helix-turn-helix transcription factor EspR in bacteria or for some homeoproteins that exert non-cell autonomous functions in the mammalian brain. Also, Sp5 might upregulate target genes that encode secreted peptides or proteins that diffuse in the extra-cellular space and exert head inhibitory functions. Such target genes, possibly taxon-specific, remain to be identified.

Thirdly, we cannot exclude that Wnt signals, which are numerous to be emitted from the apical region are not short-range signals but rather act over long-range distances to activate HySp5 expression with lipid-binding proteins or cytonemes modulating the spread of Wnt proteins as observed in Drosophila, Xenopus and zebrafish. The inhibition of Wnt3/β-catenin signaling along the Hydra body axis might thus solely be mediated by transcriptional repression, with Sp5 regulating its own expression and tightly tuning the level of Wnt signals. As a fourth divergence with the Gierer-Meinhardt model, we found that HySp5 activates its own promoter. Both the reporter assays and the ChIP-qPCR data demonstrate that HySp5 directly binds its own promoter, while the ChIP-seq data also suggest that HySp5 is able to bind the human Sp5 promoter. These observations are consistent with a study showing that the mouse Sp5 protein directly binds and activates its own promoter. In addition, β-catenin slightly enhances the activating effect of HySp5 on its promoter, likely through direct interaction between HySp5, TCF1 and/or β-catenin as observed in vitro. A recent study demonstrates a direct interaction between the zinc finger domain of mouse Sp5 and the HMG domain of Tcf/Lef1, while no direct interaction was observed for β-catenin. Also the formation of active Tcf/Lef1-β-catenin complexes appears necessary for Sp5 DNA-binding in mouse ESCs. In contrary, in human ESCs, Sp5 could directly repress the human Sp5 promoter. Thus, currently we cannot exclude that besides its auto-activating effect, HySp5 might also have an auto-repressing effect when it reaches high intracellular levels for example. Further studies should evidence this putative auto-repressing effect as well as the interactions between HySp5 and TCF/β-catenin that favor the switch from Sp5 auto-activation to Sp5 auto-repression.

### Methods

**Animal culture and drug treatment.** All experiments were carried out with Hydra vulgaris (Hv) from the Basel, AEP or Hm-105 strains. Cultures were maintained in Hydra Medium (HM: 1 mM NaCl, 1 mM CaCl₂, 0.1 mM KCl, 0.1 mM MgSO₄, 1 mM Tris pH 7.6) or in Volvic water, supplemented with 0.5 mM CaCl₂. Animals were fed two to three times per week with freshly hatched Artemia nauplii and starved for four days before any experiment. For drug treatments Hv_Basel were treated for two days with 5 μM Alsterpaullone (ALP, Sigma) diluted in HM, 0.015% DMSO then rinsed 3x in fresh HM. All animals were selected randomly for experiments.

**Generation of the HyWnt3GFP-HyActdsRED transgenic strain.** To induce gametogenesis, H. vulgaris of the strain AEP were fed with freshly hatched Artemia nauplii 7 days per week for three weeks and then 1x per week for 1 week. Thereafter, male and female animals were cultured together, resulting in fertilized embryos. The hoTG-HyWnt3FL-EGFP-HyActdsRED plasmid (kind gift from T. Holstein, Heidelberg) was injected into one-cell stage embryos. Out of 504 injected eggs, 104 embryos hatched and 7/104 embryos exhibited GFP fluorescence in the hypostome.

**RNA interference.** In short, intact Hydra were briefly washed and incubated for 45 min in Milli-Q water. 20 animals per condition were placed in 200 μl 10 mM sterilized HEPES solution (pH 7.0) and then transferred into a 0.4 cm gap electroporation cuvettes (Cell Projects Ltd). Animals were electroporated with 4 μl of 100 nM of Sp5 (siRNA-1 siRNA-2 siRNA-3) or scramble siRNAs (Supplementary Table 1b) using the Biorad GenePulser Xcell electroporation system. For double knockdown experiments 2 μM of Sp5 siRNAs were mixed with 2 μM of scramble
β-catenin siRNAs. The conditions of electroporation were: Voltage: 150 Volts; Pulse length: 50 milliseconds; Number of pulses: 2; Pulse interval: 0.1 s. For subsequent RNAi experiments, DNA constructs were kept in 18 h at RT unless indicated otherwise, incubated in 0.2x SSC, 0.1% Tween, 2 × 10 min in MAB-Buffer1 (1× MAB, 0.1% Tween), 19 h at 58 °C. Next, the samples were rinsed 3x in pre-warmed PostHyb-1 (50% Dextran) containing 200 ng DIG-labeled riboprobe was heated 5 min at 80 °C, then fixed in 2% urethane/HM for one minute, the aggregates were kept for one hour at 18 °C in 75% DM/HM and over-night in 50% DM/HM. On the next day, the aggregates were transferred into HM.

Reaggregation. Animals were electroporated twice (RNAi1, RNAi2) with siRNAs and treated with ALP as described above. Next, 50–60 animals of the same size that did not show any phenotypic signs were dissociated in 10 mL of dissociation medium (DM) (3.6 mM KCl, 6 mM CaCl2, 1.2 mM MgSO4, 6 mM Na-Citrate, 6 mM Pyruvate, 4 mM Glucose and 12.5 mM TES; pH 6.9) and the cell suspension was centrifuged at 1,400 rpm for 30 min at 4 °C. The pellet was resuspended in 0.5 mL DM, and 450 μL of the cell suspension was equally distributed into 1.5 mL tubes, followed by centrifugation at 1,400 rpm for 30 min. After detachment, the aggregates were kept for one hour at 18 °C in 75% DM/HM and over-night in 50% DM/HM. The aggregates were then transferred to a white OptiPlate™-96 (PerkinElmer) and measured with a multi-label detection platform (CHAMELEON™).

 Reporter assays in human HEK293T cells. HEK293T cells were maintained in DMEM High Glucose, 1 mM Na pyruvate, 6 mM L-glutamine, 10% fetal bovine serum. For the luciferase assays HEK293T cells were seeded into 96-well plates (5000 cells/well) and transfected 18 h later with X-tremeGENE™ HP DNA transfection reagent (Roche). The plasmids listed in Supplementary Table 2 were transfected as follows: pGL2-β-gal (Invitrogen) for control, HySp5 (Promega) for the HySp5 promoter, HySp5-ΔDBD and HySp5-ΔSP-ΔDBD constructs were generated using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, CA) following the manufacturer’s instructions. To generate the ZfSp5-ΔDBD construct, the ZfSp5-FL plasmid sequence was PCR-amplified except the DNA-binding domain. For preparing riboprobes, the HyWnt3, HyBra1, HyTsp1, HyKazal1 and HySp5 PCR products were cloned into pgEM-T-Easy (Promega). All constructs were verified by sequencing. All plasmids are listed in Supplementary Table 2 and primer sequences in Supplementary Table 1a.

ChIP-seq sample preparation. 92’000 HEK293T (92 cells/μL) cells were seeded into a 10 cm dish containing 10 mL of cell culture medium and transfected as described above with HySp5 or ZfSp5a, both containing a C-terminal HA tag (3’666 ng). Twenty-four hours later, cells were collected, washed twice in pre-warmed culture medium, fixed in 1% formaldehyde (FA) solution (Sigma) for 15 min until Glycine was added (final 125 mM) for 3 more minutes. In subsequent steps numerous reagents were from Active Motif™ (AM). The cells were washed once in ice-cold PBS and re-suspended in 5 mL chromatin prep buffer (AM), containing 0.1 mM PMSF and 0.1% protease inhibitor cocktail (PIC). The sample was transferred into a pre-cooled 15 mL glass tube, frozen with 30 strokes and incubated on ice for 10 min. Nuclear were centrifuged at 1250 for 5 min at 4 °C, resuspended in 500 μL sonication buffer (1% SDS, 50 mL Tris-HCl pH 8, 0.10 mM EDTA pH 8, 1 mM PMSF, 1% PIC), incubated on ice for 10 min. Next, the chromatin was sonicated with a BioBlock Scientific VibraCell 750402 sonicator (Amplitude: 25%, Time: 12 min, 30 s on, 30 s off, 24 cycles in) in conditions optimized to have a fragmentation size of ~250 bp. Then 100 μL of the sonicated chromatin was added to 900 μL ChIP dilution buffer (0.1% NP-40, 0.02 M HEPES pH 7.3, 1 mM EDTA pH 8.0, 0.15 M NaCl, 1 mM PMSF, 1% PIC) and incubated with 4 μg anti-HA antibody overnight at 4 °C on a rotor. Next, the sample was loaded on a X-treme-PT Protein A Superflow Q sepharose column (AM) (5:1 volume) pre-washed with 4 h on a rotator, washed 6x with 1 mL AM1 buffer and the DNA eluted with 180 μL pre-warmed AM4 buffer. The sample was decrosslinked by adding 100 μL high salt buffer (1 M NaCl, 3x TE buffer) and incubated for 5 h at 65 °C. RNAase A (10 μg/μL) was added and the sample incubated at 37 °C for 30 min before adding PK (10 ng/μL) and further incubated for 2 h at 55 °C. The DNA was purified with the MiniElute PCR purification kit (Qiagen). For preparing the Input DNA, 5 μL sonicated chromatin was diluted in 45 μL 0.5 M NaCl, incubated for 15 min at 95 °C, then transferred to 37 °C, incubated for 5 min with RNAase A (10 μg/μL) and PK (10 ng/μL) and incubated at 55 °C for 30 min. 10 μL were taken for purification (MiniElute PCR purification kit from Qiagen).

RNA-seq sample preparation. 156’500 HEK293T (78.25 cells/μL) cells were seeded into a 6-well plate containing 2 mL of cell culture medium and transfected as described above with 626 ng of HySp5, ZfSp5a, HySp5-ΔDBD, ZfSp5a-ΔDBD and 313 ng of human Aβ-CAT. RNA was extracted using the E.Z.N.A. total RNA kit I from OMEGA following the manufacturer’s instructions.

Co-immunoprecipitation assay and Western blotting. 92’000 HEK293T cells (92 cells/μL) were seeded into a 10 cm dish containing 10 mL of cell culture medium and transfected as described above with 626 ng of HySp5, ZfSp5a, HySp5-ΔDBD, ZfSp5a-ΔDBD and 313 ng of human Aβ-CAT. RNA was extracted using the E.Z.N.A. total RNA kit I from OMEGA following the manufacturer’s instructions.

Plasmid constructions. To generate the HyWnt3Luc construct 2149 bp of the Hydra Wnt3 promoter were transferred from the huTG-HyWnt3EL-EGFP construct (kind gift from T. Heinzelin, Heinzelin lab) into the pRC303 reporter construct (kind gift from Z. Kozmick, Prague) 29. For the HyWnt3ΔR-ΔEpLuc construct, the whole HyWnt3Luc plasmid sequence was PCR-amplified except the 386 bp corresponding to the repressor element. For the JIΨWnt3Luc construct 3997 bp of the zebrabfish Wnt3 promoter were transferred from pZEGF-Wnt3 (kind gift of Cathleen Teh, Singapore) into pG3.L. For the HySp5Luc construct, 2992 bp of the Hydra Sp5 promoter were PCR-amplified from Hm-105 genomic DNA and sub-cloned into pG3.L. To express HA-tagged HySp5, ZfSp5a, ZfSp5+1 proteins, a C-terminal HA-tag was introduced into the pCS2 + constructs encoding the Hydra Sp5 (human codon-optimized), zebrabfish Sp5a and Sp5 full-length coding sequences. The HySp5-ΔSP construct was produced by inserting a human codon-optimized HySp5 sequence lacking 110 amino acids of the N-terminal end together with a C-terminal HA-tag into pCS2+. The HySp5-ΔDBD and HySp5-ΔSP-ΔDBD constructs were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, CA) following the manufacturer’s instructions. To generate the ZfSp5a-ΔDBD construct, the ZfSp5a-FL plasmid sequence was PCR-amplified except the DNA-binding domain. For preparing riboprobes, the HyWnt3, HyBra1, HyTsp1, HyKazal1 and HySp5 PCR products were cloned into pGEM-T-Easy (Promega). All constructs were verified by sequencing. All plasmids are listed in Supplementary Table 2 and primer sequences in Supplementary Table 1a.

Western blotting. Protein extracts were prepared as described before 30. The aggregates were kept for one hour at 18 °C in 75% DM/HM and over-night in 50% DM/HM. On the next day, the aggregates were transferred into HM.

Peroxidase assay. Hydra were relaxed in 2% urethane/HM for one minute and fixed in 4% PFA prepared in HM (pH 7.5) for 2 h at RT. Samples were washed 3 × 10 min with PBS, followed by adding 500 μL DAB (SIGMAFAST™ 3.3’- Diamino-benzidine) solution. The DAB solution was prepared as follows: 1 tablet of DAB was dissolved in 10 mL of PBS and filtered with a 0.2 μm filter. 5 μL of the filtered solution was added to 5 mL of PBS together with 20 μL of Triton X-100 (0.2%) and 1 μL of a 3% H2O2 solution. The animals were incubated for 10 min in DAB solution and the reaction stopped by washing the samples 3 × 10 min with PBS.

Peroxidase assay. Hydra were relaxed in 2% urethane/HM for one minute and fixed in 4% PFA prepared in HM (pH 7.5) for 2 h at RT. Samples were washed 3 × 10 min with PBS, followed by adding 500 μL DAB (SIGMAFAST™ 3.3’-Diamino-benzidine) solution. The DAB solution was prepared as follows: 1 tablet of DAB was dissolved in 10 mL of PBS and filtered with a 0.2 μm filter. 5 μL of the filtered solution was added to 5 mL of PBS together with 20 μL of Triton X-100 (0.2%) and 1 μL of a 3% H2O2 solution. The animals were incubated for 10 min in DAB solution and the reaction stopped by washing the samples 3 × 10 min with PBS.
supplemented with 1 mg/ml BSA, 3x in 500 μL of Co-IP wash buffer supplemented with 300 mM NaCl. The column was centrifuged at 1250 g x 3 for 5 min and 25 μL 2x reduction buffer was added. The resulting eluate was used to determine its concentration. After centrifugation at 1250 g x 5 μL glycera (Sigma) was added and the sample was boiled for 5 min at 95 °C before loading on a 8% SDS-PAGE gel, electrophoresed and transferred onto PVDF membrane (Bio-Rad). Then all steps were performed at RT unless specified. The membrane was blocked in M-TRS-Tw (TRS containing 0.1% Triton X-100) for 0.5 h before primary antibodies were applied for 1 h. The membrane was washed 4 × 10 min in TBS-Tw, incubated in anti-rabbit (ab99697, Abcam) or anti-mouse (W402B, Promega) IgG horseraddish peroxidase antibody (1:5000) for one hour, visualized with Western Lightning® Plus-ECL reagent (PerkinElmer). 10 μg extract were used as input sample. Antibodies: anti-HA antibody (NB600–363, Novus Biologicals), anti-β-catenin antibody (610153, BD Biosciences), anti-TCF1 (sc-271453, Santa Cruz Biotechnology). All uncropped western blots can be found in Supplementary Fig. 15.

ChiP-seq data analysis. Demultiplexed ChiP-seq reads from our sequenced samples were mapped onto the Human GRCh37 (hg19) genome assembly using bowtie2, version 2.2.6.28, implemented in galaxy.27 Significantly enriched regions were identified using MACS2 (version 2.1.0.151222.0). Coverage files were normalized by the millions of mapped reads in each sample using a manually created R script. Normalized bedgraph files were converted to bigwig using the WigToBedGraph-to-bigWig converter tool (version 1.1.1) implemented in the public Galaxy server (https://usegalaxy.org/) and visualized with UCSC genome browser. The fastq files from the two biological replicates of each condition were merged and normalized. Normalized bedgraph files were used to obtain the average coverage profile at autosomal chromosomes were analysed in this study. MACPeaks regions were either extended or cropped from their respective center to match a 100 kb window.

ChIP-seq data analysis. Demultiplexed RNA-seq reads from our sequenced samples were mapped onto the Human GRCh37 (hg19) genome assembly using the STAR RNA-seq aligner.62 Work implemented in Galaxy. The fastq files from the three biological replicates of each condition were merged and remapped in order to obtain the average coverage profile. Coverage files were normalized by the millions of mapped reads in each sample using a manually created R script. Normalized bedgraph files were converted to bigwig using the WigToBedGraph-to-bigWig converter tool implemented in the public Galaxy server (https://usegalaxy.org/) and visualized with UCSC genome browser. We used HTSeq63 implemented in R script to scan the MACPeaks regions for the Sp5 and β-catenin occupancies in mouse ES cells31,61 were, up- and downregulated genes were sampled independently to extract DNA material using the DNAeasy Blood & Tissue kit (Qiagen). Sequencing libraries were prepared using the TruSeq Nano DNA kit (Illumina), with 350 bp insert sizes, and sequenced paired-end using 150 cycles on an Illumina HiSeq X Ten sequencer by Macrogen Inc. Average and standard deviations of insert sizes of the sequenced reads were measured using 10 mio reads mapped to a preliminary assembly of each genome, then the two genomes were assembled using MaSuRCA v3.2.1.199. All scaffolds (>300 bp) and unplaced contigs (≥500 bp) were retained in the final set of sequences. The redundancy of each assembly was reduced by using CD-HIT-est v4.9.27 with a 100% identity threshold. Sequencing depth was evaluated from the number of reads and expected genome length: Hydra viridissima: 120×; Hydra oligactis: 50×. Scaffolds assembly statistics in bp: number of scaffolds: 85677 for viridissima and 447337 for oligactis; N50: 11871 for viridissima and 5391 for oligactis.

Hydra RNA-seq transcriptomics. For spatial and cell-type RNA-seq transcriptomics, see ref. 32. All profiles publicly available on the HydraATLAS server (https://HydraATLAS.unige.ch).

Multiple sequence alignment and phylogenetic analysis. For Supplementary Figure 2, the multiple sequence alignment was generated using T-Coffee.21 The conserved zinc fingers domains, SP and SP6 boxes were visualized by IBS.22 For the phylogenetic analysis of the Sp5, Sp-related and Klf-related gene families (Supplementary Figure 3), sequences from Hydra as well as from other cnidarian, ecdysozoans, lophotrochozoans and deuterostomes representative species were retrieved from Uniprot or NCBI, aligned with Muscle align (www.ebi.ac.uk/Tools/msa/muscle/) or MAFFT (https://mafft.ebi.ac.uk/alignment/server/) and tested in iterative PhyML 3.0 analyses using the LG substitution model, 8 substitution rate categories and 100 bootstrap23.

SP5 expression in zebrafish embryos. For all zebrafish experiments, colonies of the strain AB-Tu or Nacre were used, with animals maintained at 28 °C with a maximal density of five fish per liter in a 14 h light–10 h dark cycle. The fish were fed twice a day with 2-day-old Artemia and fish embryos incubated at 28 °C. For overexpression experiments, capped sense mRNAs were synthesized using the message mMachine™ Transcription Kit from Ambion (Ambion, Austin, TX USA) and 400 pg of HySp5, HySp5-ΔDBD, HySp5-ASP or HySp5-ASP-ΔDBD mRNAs injected into one cell stage embryos. For mRNA co-injection experiments, injected amounts were as follows: 400 pg of HySp5 and 4 pg of ZWnt8 mRNA. All embryos were scored for phenotypes 48 h post fertilization.

Statistical analyses. All statistical analyses were performed with the software GraphPad Prism7. The statistical tests were two-tailed unpaired.

Hydra RNA-seq transcriptomics. For spatial and cell-type RNA-seq transcriptomics, see ref. 32. All profiles publicly available on the HydraATLAS server (https://HydraATLAS.unige.ch).

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Data availability

The HySp5 sequence has been deposited in GenBank under: MG437301. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The Source Data underlying Figs. 4f, 5d and Supplementary Figures 6b, 12d are provided as a Source Data file.

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Author contributions
M.C.V. performed Hydra and cell culture experiments, performed biochemical assays and prepared ChIP-seq and RNA-seq samples; L.B. analyzed ChIP-seq and RNA-seq data. M.C.V. and L.B. performed ChIP-qPCRs. L.I.O. contributed to plasmid constructions, knockdown experiments and in situ hybridizations; C.R. and S.V. performed zebrafish experiments; Y.W. and B.G. designed the Hydra high-throughput transcriptomics, Y.W. produced and processed the Hydra high-throughput transcriptomics as well as the genome data; C.P. produced the transgenic line; M.C.V. and B.G. conceived the study, M.C.V., L.B. and B.G. wrote the manuscript.

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