Autoregulatory and repressive inputs localize *Hydra Wnt3* to the head organizer

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Polarized Wnt signaling along the primary body axis is a conserved property of axial patterning in bilaterians and prebilaterians, and depends on localized sources of Wnt ligands. However, the mechanisms governing the localized *Wnt* expression that emerged early in evolution are poorly understood. Here we find in the cnidarian *Hydra* that two functionally distinct *cis*-regulatory elements control the head organizer-associated *Hydra Wnt3* (*HyWnt3*). An autoregulatory element, which mediates direct inputs of Wnt/β-catenin signaling, highly activates *HyWnt3* transcription in the head region. In contrast, a repressor element is necessary and sufficient to restrict the activity of the autoregulatory element, thereby allowing the organizer-specific expression. Our results reveal that a combination of autoregulation and repression is crucial for establishing a Wnt-expressing organizing center in a basal metazoan. We suggest that this transcriptional control is an evolutionarily old strategy in the formation of Wnt signaling centers and metazoan axial patterning.

regulatory DNAs | *cis*-regulation | enhancer | molecular evolution | axis formation

The establishment and patterning of the primary body axis is fundamental to metazoan body plan development, a conserved feature of which is the spatially restricted expression of *Wnt* genes at the posterior end (1, 2). In cnidarians and bilaterians, *Wnt* genes are expressed in the blastopore and equivalent regions, and this localized *Wnt* expression is critical for organizing the primary body axis (1–3). Polarized *Wnt* expression in the sponge *Amphimedon* embryo (4) provides evidence that the origin of the Wnt signaling center is at the base of metazoan evolution. However, the regulation of localized *Wnt* expression is largely unknown.

In basal metazoans, the axial role of Wnt/β-catenin signaling has been extensively studied in the cnidarian freshwater polyp *Hydra* (5–9), which has a single body axis, the oral–aboral axis, with a head at the oral end and a foot at the aboral end. The head of the adult *Hydra* is classically defined to consist of the upper part carrying the hypostome, a domelike structure with the mouth opening in its center, and the lower part with the tentacles. Axial patterning in *Hydra* is controlled by the head organizer, located in the apical tip of the hypostome (10, 11). It has been hypothesized that the organizer patterns the body along the oral–aboral axis through diffusible short-range autocatalytic activators and long-range inhibitors (12, 13). Although the molecular identity of these theoretical factors is still uncertain, Wnt/β-catenin signaling has been postulated to encompass the activator (3).

Hydra Wnt (*HyWnt*) genes are expressed at the apical tip of the hypostome, whereas the transcriptional components of Wnt/β-catenin signaling *Hydra Tcf* (*HyTcf*) and nuclear *Hydra* β-catenin (Hyβ-catenin) are more broadly distributed along the oral–aboral axis, with higher levels in the hypostome than in the body column (5–7, 14). These genes are also induced when a new head organizer is formed during asexual reproduction by budding and during head regeneration (5). The sufficiency of Wnt/β-catenin signaling for providing the head organizer activity has been recently demonstrated by overexpression of a constitutively active form of *Hyβ-catenin* (9).

A putative master Wnt ligand in *Hydra* axial patterning is *HyWnt3*, being expressed at the earliest phase of head regeneration and stimulating head organizer formation (7). The coup-regulation of *HyTcf* and *Hyβ-catenin* during head organizer formation suggests that they are involved in *HyWnt3* transcriptional regulation. Indeed, elevated activation of Wnt/β-catenin signal induces ectopic *HyWnt3* expression (6), and autoregulation through the Wnt/β-catenin circuit has been proposed to activate and maintain *HyWnt3* expression (6), although a direct molecular evidence is missing. A simple autoregulation although cannot explain the restriction of *HyWnt3* expression to the head organizer, as *HyTcf* and *Hyβ-catenin* are broadly expressed and seem to be required for the narrower *HyWnt3* expression (3, 5, 15). Thus, the mechanism for *HyWnt3* regulation remains to be discovered.

By extensively using transgenic *Hydra*, we analyzed *cis*-regulation of *HyWnt3*. We identified two functionally distinct *cis*-regulatory elements in the *HyWnt3* promoter that are responsible for the head organizer-specific *HyWnt3* expression. An autoregulatory element interacts with the HyTcf/Hyβ-catenin transcriptional complex and induces gene expression in a broad domain in the head. By contrast, a repressor element is necessary and sufficient for restriction of the expression to the head organizer. These results demonstrate that a combination of autoregulation and repression has a crucial function for the establishment and maintenance of the localized *HyWnt3* expression in the head organizer.

Results

***HyWnt3* Upstream Promoter Sequence Reproduces the Endogenous Expression in the Head Organizer Region.** To dissect the molecular mechanisms regulating *HyWnt3* expression in the head organizer, we sought to identify *HyWnt3 cis*-regulatory elements. A 2,149-bp fragment (HyWnt3FL) upstream of the *HyWnt3* translation initiation site was isolated and analyzed for its ability to drive expression in transgenic *Hydra* polyps. Injection of the HyWnt3FL with an EGFP reporter gene (*HyWnt3FL-EGFP*) into *Hydra* embryos produced transgenic polyps, in which EGFP expression was exclusively observed at the apexes of the adult hypostome and developing buds, reflecting the endogenous expression of *HyWnt3* (Fig. 1 *A* and *B*). As demonstrated by in situ hybridization the reporter expression was also activated in the apical tip of head-regenerating animals (Fig. 1 *C* and *D*), in which the head organizer is restored. EGFP fluorescence signals were not detectable in regenerating tips as a result of the delay in protein maturation. Previous studies showed that *HyWnt3* is activated by high Wnt/β-catenin signaling caused by alsterpaullone

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DEVELOPMENTAL BIOLOGY

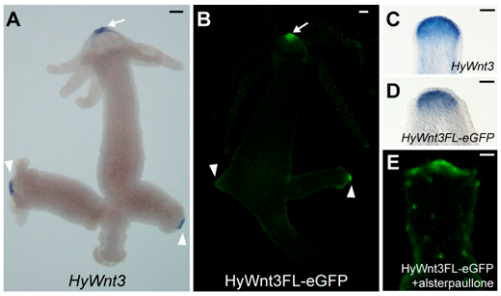

Fig. 1. *HyWnt3* promoter reproduces the endogenous expression. (*A* and *B*) Expression of *HyWnt3* mRNA (*A*) and *HyWnt3FL-EGFP* transgene (*B*) in the adult *Hydra*. *HyWnt3FL-EGFP* is activated exclusively in the *HyWnt3*-expressing cells in the apical tips of the adult hypostome (arrow) and developing buds (arrowheads). (*C* and *D*) Induction of *HyWnt3* and *HyWnt3FL-EGFP* in head-regenerating animals at 3 h after head removal, visualized by in situ hybridization for *HyWnt3* (*C*) and *EGFP* (*D*). (*E*) Ectopic activation of *HyWnt3FL-EGFP* reporter in the body column at 48 h after alsterpaullone treatment for 24 h. (Scale bars: 100 μm.)

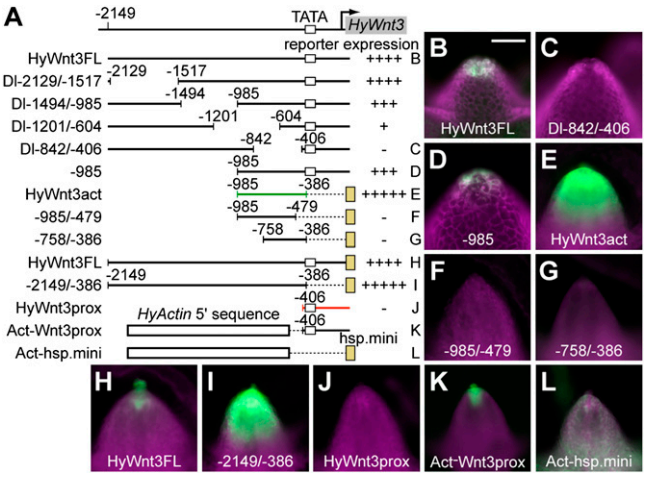

Fig. 2. Identification of HyWnt3act and HyWnt3rep *cis* elements for *HyWnt3* expression. (*A*) Schematic diagram of deletion constructs used for the identification of the HyWnt3act (green) and HyWnt3rep (red) elements. The numbers indicate positions from the translation start site. Reporter expression activities of the constructs are indicated (*Right*). (+++++, Augmented levels of expression; ++++, WT levels of expression; +++, moderately reduced expression; ++, severely reduced expression; +, expression detectable; –, expression undetectable.) The white and yellow boxes indicate putative TATA boxes of the *HyWnt3* promoter (TATA; −378, −368, and −360 bp) and the *Hyhsp70* minimal promoter (hsp.mini), respectively. (*B–L*) EGFP expression in the hypostome of transgenic animals with the reporter construct HyWnt3FL (*B* and *H*), Dl−842/−406 (*C*), −985 (*D*), HyWnt3act (*E*), −985/−479 (*F*), −758/−386 (*G*), −2149/−386 (*I*), HyWnt3prox (*J*), Act-Wnt3prox (*K*), and Act-hsp.mini (*L*). Ectodermal or endodermal lines are shown in *B–D* or *E–L*, respectively. Reporter constructs lacking the *HyWnt3* proximal promoter sequence exhibit dramatic expansion of expression (*E* and *I*). In contrast, reporter constructs involving it displayed restricted expression to the head organizer (*B*, *D*, *H*, and *K*). (Scale bar: 100 μm.)

treatment, which inhibits the activity of glycogen synthase kinase-3β (GSK-3β) involved in β-catenin degradation in *Hydra* (6). In agreement with the endogenous responsiveness, *HyWnt3FL-EGFP* was ectopically activated in multiple spots along the body column following alsterpaullone treatment (Fig. 1*E*). Thus, in all experimental settings, *HyWnt3FL-EGFP* activity mimicked endogenous *HyWnt3* expression, demonstrating that HyWnt3FL contains the regulatory elements required for *HyWnt3* expression in the head organizer.

***HyWnt3* Regulatory Region Consists of Activator and Repressor Modules.** To identify the sequences within HyWnt3FL responsible for the head organizer-specific expression, a series of internal deletions of HyWnt3FL was generated and analyzed for their activity in transgenic polyps (Fig. 2 and Figs. S1 and S2). The deletion of −2,129 to −985 bp did not significantly change reporter expression (Dl−2129/−1517 and Dl−1494/−985; Fig. 2*A* and Fig. S2). In contrast, removal of −1,201 to −604 bp (Dl−1201/−604) strongly reduced reporter expression, and deletion of −842 to −406 bp (Dl−842/−406) eliminated expression (Fig. 2 *A* and *C*). Generation of multiple transgenic lines for each of the deletion construct and an independent transformation marker *Hydra Actin* (*HyActin*)–*red fluorescent protein* (*RFP*) reporter gene in the same construct ensured that the hypostome cells carried the *HyWnt3 EGFP* reporter gene under examination (Fig. 2 and Fig. S1). Thus, the −1,201 to −406 bp region contains the sequences essential for *HyWnt3* expression in the head organizer. Further, the −985 bp upstream sequence (−985) drove reporter expression at the apex of the hypostome (Fig. 2*D*), indicating that the crucial sequences for *HyWnt3* transcription are located in the −985 to −406 bp region.

We next tested whether a fragment encompassing the −985 to −406 bp region was sufficient to drive expression in the hypostome. The −985 to −386 bp fragment, which we named HyWnt3act enhancer element, was linked to the core promoter from the *Hydra heat shock protein 70* (*Hyhsp70*) gene (hsp.mini) and *EGFP* reporter gene, and analyzed for its activity. The hsp.mini was demonstrated to be a functional core promoter in our heterologous reporter analysis (Fig. S1). The HyWnt3act element induced reporter expression in the hypostome (Fig. 2*E*). Surprisingly, HyWnt3act also directed ectopic reporter expression in the lower part of the head, whereas HyWnt3FL activity was limited to the tip of the hypostome when examined using the hsp.mini reporter construct (Fig. 2*H*). This observation indicates that the downstream region of −386 bp is necessary to limit *HyWnt3* expression to the head organizer region (as detailed later). We also noticed that the HyWnt3act element is active only in endodermal epithelial cells, indicating that the *HyWnt3* expression in ectodermal cells also requires the downstream region of −386 bp because −985 drove reporter expression both in the ectoderm and endoderm (Fig. 2 and Fig. S2). Loss of reporter activity of −985/−479 and −758/−386 demonstrated that HyWnt3act was a minimal enhancer in our studies (Fig. 2 *F* and *G*).

The *cis*-regulatory analysis of HyWnt3act suggested a possible negative regulation of *HyWnt3* by the proximal sequence of HyWnt3FL. Indeed, the −2,149 to −386 bp fragment (−2149/−386), which included the entire 5′ sequence of HyWnt3FL but lacked the proximal sequence (Fig. 2*A*), drove similarly increased reporter expression (Fig. 2*I*). Thus, the proximal sequence of HyWnt3FL seems to be essential for *HyWnt3* repression outside of the head organizer region. A −406 bp *HyWnt3* proximal promoter region (HyWnt3prox) on its own did not drive reporter expression (Fig. 2*J*). However, we speculated that, if HyWnt3prox is responsible for *HyWnt3* repression in cells outside of the head organizer region, it might be capable of repressing the activity of a given promoter that is normally active outside of the head organizer. To address this, we made use of the *HyActin* promoter, which is active in the entire animal (16). When the *HyActin* 5′ sequence (−1,300 to −221 bp *HyActin* promoter lacking its putative core promoter) was fused to the HyWnt3prox sequence (Act-Wnt3prox; Fig. 2*A*), this chimeric fragment activated reporter expression only in the apical end of the hypostome (Fig. 2*K*), which is the same pattern as that resulting from HyWnt3FL (Fig. 2*H*). In combination with other heterologous promoters (e.g., the *Hyhsp70* core promoter), the *HyActin* 5′ sequence (Act-hsp.mini) directed uniform expression as the full-length

HyActin promoter does (Fig. 2*L*). This result demonstrated that HyWnt3prox was compatible with the *HyActin* 5′ regulatory activity, but was able to induce reporter expression only in the head organizer. We therefore conclude that HyWnt3prox has a repressive function on gene expression in cells distant from the head organizer, and *HyWnt3* expression can be locally restricted exclusively by this repressor element (HyWnt3rep).

HyWnt3act Requires Direct Inputs by T Cell-Specific Factor and Other Transacting Factors. An autoregulatory positive feedback loop of Wnt/β-catenin signaling has been previously suggested to control *HyWnt3* expression (5, 6, 14). To investigate whether HyWnt3act is under direct Wnt/β-catenin signaling control, we surveyed HyWnt3act for T cell-specific factor (TCF) binding sites and found three candidate sites (Fig. 3*B*). To examine whether Tcf protein binds to these sites, we performed EMSA (Fig. 3*A*). Each of the three sites showed binding affinity for recombinant *Xenopus* Tcf-3 (XTcf-3). This XTcf-3 binding was inhibited by competition with a TCF-binding site from the *Xenopus siamois* promoter, which was confirmed to interact with XTcf-3 (17). Consistently, mutation of the TCF sequences in the probe abolished binding, demonstrating a sequence-specific interaction of recombinant XTcf-3 with the TCF sites from HyWnt3act enhancer.

Having defined the TCF sites as potential binding sequences for transcriptional activation of *HyWnt3*, we tested their function in vivo. We introduced mutations into the TCF-binding sequences defined by EMSA and analyzed the effects on the HyWnt3act enhancer activity in transgenic *Hydra* (Fig. 3 *B–F*). Simultaneous mutation of all three TCF-binding sites (mTCFall) resulted in complete loss of reporter activity (Fig. 3*C*). Subsequently, individual mutations of the TCF sites revealed that the sites 2 and 3 are essential for the HyWnt3act function (Fig. 3 *D–F*). Thus, we concluded that *HyWnt3* expression requires TCF binding sites 2 and 3 located in the HyWnt3act enhancer for Tcf-dependent induction.

Importantly, the −758/−386 fragment, which lacks a 5′ 227-bp region of HyWnt3act but retains the TCF sites 2 and 3, failed to drive reporter expression (Fig. 2*G*), indicating that other transactivators may interact with the 5′ end of HyWnt3act and act in concert with Tcf to control the expression of *HyWnt3*. A bioinformatic analysis of the 227-bp region reveals the existence of further putative transcriptional activators, e.g., CREB/CRE, Forkhead, and LIM (Table S1). CREB/CRE binding proteins have been reported to be activated early on during head regeneration (18–20), and they are therefore potential coactivators of *HyWnt3*.

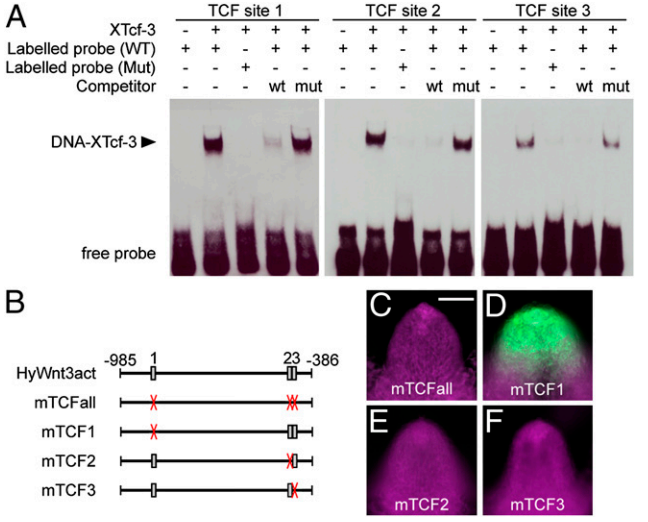

Fig. 3. Direct regulation of HyWnt3act by Tcf. (*A*) EMSA shows binding of recombinant His-tagged XTcf-3 protein to the Tcf sites 1, 2, and 3. (*B*) Schematic diagram of the HyWnt3act element with the location of TCF-binding sites indicated. Boxes and X (red) indicate WT and mutant sites, respectively. (*C–F*) Requirement of the Tcf sites in *Hydra* in vivo. Mutation of all Tcf sites (*C*) abolished reporter activity. Tcf sites 2 (*E*) and 3 (*F*), but not 1 (*D*), were essential for the activity. (Scale bar: 100 μm.)

β-Catenin Is a Direct Regulator of *HyWnt3*. Physical interaction of Hyβ-catenin and HyTcf in vitro has been demonstrated previously (5, 8), suggesting that Hyβ-catenin is recruited to TCF-binding sequences to activate transcription in cooperation with HyTcf. To test whether this mechanism also applies to the control of *HyWnt3* expression, we generated a *Hyβ-catenin-EGFP* transgenic *Hydra*, in which expression of EGFP-tagged Hyβ-catenin is driven by the *Hyβ-catenin* promoter (*Hyβ-catenin-EGFP*; Fig. 4*A*). In these animals, Hyβ-catenin-EGFP colocalized with the native Hyβ-catenin, and consistent with previous experiments using anti–Hyβ-catenin antibody (14), nuclear accumulation of Hyβ-catenin-EGFP was higher in the hypostome than in the body column (Fig. 4 *B* and *C*). Alsterpaullone treatment led to stabilization and increased nuclear accumulation of the Hyβ-catenin-EGFP throughout the animal (Fig. 4*D*), similar to the behavior of endogenous Hyβ-catenin.

To test whether Hyβ-catenin is present at the identified TCF-binding sites in vivo, we performed ChIP assay using the *Hyβ-*

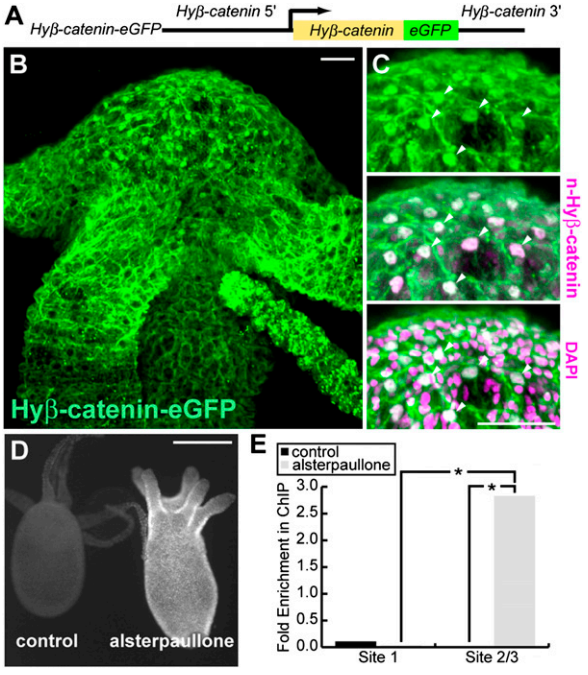

Fig. 4. Hyβ-catenin signaling directly regulates *HyWnt3*. (*A*) Schematic diagram of the *Hyβ-catenin-EGFP* construct. (*B* and *C*) Distribution of Hyβ-catenin-EGFP protein. (*B*) Hyβ-catenin-EGFP is associated with the cell membranes and localized in the nuclei of cells at higher levels in the hypostome than in the body column. (*C*) Hyβ-catenin-EGFP is colocalized with the endogenous Hyβ-catenin detected with antinuclear Hyβ-catenin antibody, in the nuclei of cells at the hypostome (arrowheads). (*D*) Hyβ-catenin-EGFP was stabilized and accumulated throughout the animal upon alsterpaullone treatment. *Hyβ-catenin-EGFP* transgenic animals were treated without (control) and with alsterpaullone for 24 h. (*E*) ChIP analysis detecting interaction of Hyβ-catenin and the *HyWnt3* promoter. The fragment containing sites 2/3 was enriched in ChIP of alsterpaullone-treated *Hyβ-catenin-EGFP* transgenic animals compared with nontreated animals. In contrast, site 1 was never enriched upon treatment (*$P < 0.05$; $n = 3$). (Scale bars: 50 μm.)

catenin-EGFP polyps and an anti-GFP antibody (Fig. 4*E*). In this ChIP experiment, the TCF-binding sites 2 and 3 were analyzed as a single sequence (site 2/3) because they are closely positioned in the promoter. In intact polyps, we failed to detect enrichment of the immunoprecipitation of the fragments containing the TCF site 1 or site 2/3 (Fig. 4*E*). This probably happens because the *HyWnt3*-expressing cells in the hypostome represent only a small fraction of the epithelial cells in the animal, and this could obstruct the attempt to identify binding of Hyβ-catenin complex in the *HyWnt3* promoter. In contrast, alsterpaullone treatment led us to detect the enrichment of immunoprecipitated sites 2/3 but not site 1 (Fig. 4*E*). These results confirm the contribution of sites 2 and 3 for *HyWnt3* regulation and demonstrate the interaction of Hyβ-catenin and the TCF sites in vivo. Overall, HyWnt3act is directly regulated by the Hyβ-catenin/HyTcf transcriptional complex.

Wnt/β-Catenin Activation Suppresses HyWnt3rep Function. We next asked how HyWnt3rep is controlled within the head organizer region. Considering the localized activity of HyWnt3FL or Act-Wnt3prox to the apical end of the hypostome, the function of HyWnt3rep might be suppressed in the head organizer and such a suppression might involve factors acting in the head organizer region. A potential candidate is Wnt/β-catenin signaling itself. To examine whether HyWnt3rep is regulated by Wnt/β-catenin signaling, we treated the Act-Wnt3prox transgenic animals with alsterpaullone. In this way, ectopic *HyWnt3* expression foci are generated, as a result of ubiquitous Wnt signaling activation. Interestingly, this treatment of Act-Wnt3prox animals led to EGFP expression in a spotted pattern in the body column (Fig. 5*B*), which was very similar to the responsiveness of HyWnt3FL (Fig. 5*A*). In contrast, the HyWnt3prox did not show such an ectopic expression (Fig. 5*C*). These results suggest that the HyWnt3prox function is suppressed at high levels of Wnt signaling; thereby, the HyActin 5′ sequence activity is "de-repressed" and becomes activated in the body column. This also means that the de-repression is independent of the TCF-binding sequences within the HyWnt3act element, and thus controlled by other transcription factors. From this experiment, we conclude that HyWnt3rep is sensitive to high levels of Wnt/β-catenin signaling activation so that it becomes suppressed, thereby allowing *HyWnt3* gene expression in the head organizer region.

Discussion

How Wnt signaling centers are formed and maintained is an important but unsettled question. By using transgenic *Hydra*, we analyzed *cis*-regulatory mechanisms of *HyWnt3* transcription and found that it represents a key level in the formation of such a center. We found that in the *Hydra* head organizer *HyWnt3* is regulated by two functionally distinct *cis*-regulatory elements located in the *HyWnt3* promoter (Fig. 6). One of them, HyWnt3act, acts as an autoregulatory element mediating direct Wnt/β-catenin signaling. This demonstrates that Wnt/β-catenin signaling directly regulates *HyWnt3*. When it has been expressed, *HyWnt3* can maintain its own expression by creating a positive feedback loop (Fig. 6). This regulatory system fits well with the pattern formation model proposed by Gierer and Meinhardt (3, 12).

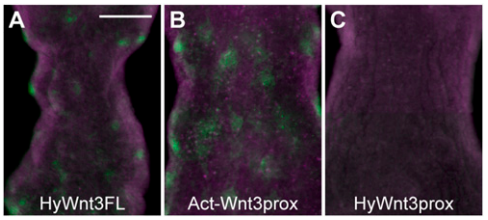

Fig. 5. Derepression of HyWnt3rep by Wnt activation. (*A*–*C*) EGFP reporter activities of transgenic *Hydra* carrying HyWnt3FL (*A*), Act-Wnt3prox (*B*), and HyWnt3prox (*C*) after 48 h for 24-h alsterpaullone treatment. Act-Wnt3prox showed ectopic EGFP expression in the body column in a spotted pattern similar to that resulting from HyWnt3FL but not HyWnt3prox. (Scale bar: 100 μm.) This figure is a composite of multiple panels.

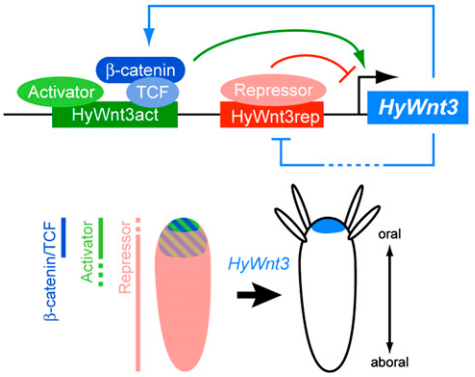

Fig. 6. Model for the transcriptional regulation of the head organizer-specific *HyWnt3* expression. Schematic diagram of the mechanistic model governing *HyWnt3* in the head organizer. *HyWnt3* is controlled by two distinct *cis*-regulatory elements, the HyWnt3act (green) and HyWnt3rep (red) elements, which are positively (light blue arrow) and negatively (light blue bar) regulated by Wnt/β-catenin signaling. The β-catenin/Tcf complex (blue) and putative activators (light green) bind to HyWnt3act, and their combinatorial inputs act in *HyWnt3* transcription (green arrow). Potential repressors (pink) bind to HyWnt3rep and inhibit *HyWnt3* expression (red bar). Presumed distribution or activity of the β-catenin/Tcf and activators is in and below the head, and that of repressors is absent from the organizer region along the body axis. Their positive and negative regulation restricts *HyWnt3* expression (light blue) to the head organizer region. Note that, in addition to the transcriptional level, there are also inhibitory inputs by HyDkk1/2/4 inhibition in the gastric region (36).

In addition to the other *Wnt* genes (7, 21), *Brachyury* and *Goosecoid* define the posterior, blastopore signaling center (22, 23) in *Hydra* and other cnidarians. There is evidence that Wnt signaling centers in bilaterians are also regulated by Wnt ligands themselves. Reminiscent of the *Hydra* hypostome, *Wnt* genes are expressed in the posterior growth zone in arthropods and vertebrates (24). It is not known yet whether the same genetic cascades act in maintaining *Wnt* expression. In zebrafish, a Brachyury/Wnt loop acts in *Wnt3a* and *Wnt8* expression in the posterior growth zone (25), which is not present in insects (26, 27). A positive feedback regulation by Wnt/β-catenin signaling has been also shown for the sea urchin *Wnt8* (28, 29), in which it is required for the maintenance of the vegetal Wnt signaling center during gastrulation. This conservation in the prebilaterian and bilaterian animals implies that the positive autoregulatory feedback machinery of Wnt/β-catenin signaling can be an ancient tool used for the maintenance of the Wnt signaling center during metazoan evolution.

Although the activation of HyWnt3act requires the Hyβ-catenin/HyTcf transcriptional complex binding to this element, HyWnt3act appears to act as a hub of autoregulatory signals and inputs of other transcriptional activators. A 5′ 227-bp deletion from the HyWnt3act fragment (−758/−386) resulted in loss of reporter expression, although the functional TCF binding sites remained intact (Fig. 2). We found that a number of potential transcription factor binding motifs exist in this region (Table S1). In particular, CBP/CREB, Forkhead domain factors, and LIM homeodomain transcription factors are potential candidate molecules. CBP is known to act as a transcriptional coactivator of β-catenin (30), and is activated during early head regeneration in *Hydra* (18, 19, 31). In *Drosophila*, *forkhead* transcription factor is required for expression of the *Drosophila Wnt1* homologue *wing-*

less in the posterior end of the embryo (32). The *Hydra* orthologue of *forkhead*, budhead, is expressed in the hypostome (33), making possible a similar genetic cascade on *HyWnt3* activation. The LIM homeobox gene functions in activation of vertebrate organizer genes (34), and recently a cnidarian LIM counterpart suggested its conserved role in organizer gene activation (35). It is possible that transcription factors that are expressed in Wnt signaling centers in both prebilaterians and bilaterians have played a role in establishing *Wnt* expression in combination with Wnt autoregulation in the evolution of the Wnt signaling center.

Given the positive autoregulation on *HyWnt3* transcriptional control, how can its expression be confined to the head organizer? Also, how can *HyWnt3* emerge as a restricted domain from the broad distribution of *HyTcf* and *Hyβ-catenin* (3, 15) despite their major contributions to the autoregulation? Reaction-diffusion models of pattern formation (12) predict that the autocatalytic activator produces a long-range inhibitor that restricts the activating source to the organizing center. The Wnt antagonist *Hydra* Dickkopf1/2/4 (HyDkk1/2/4) (36) was postulated as a potential inhibitor (37). As Dkk inhibits ligand receptor interactions, this mechanism does not explain how *Wnt* expression is inhibited on the transcriptional level.

We showed that transcriptional regulation is essential to restrict *Wnt* expression to the site of the signaling center. We discovered a HyWnt3rep repressor element that is necessary and sufficient for localizing *HyWnt3* expression to the head organizer. The removal of HyWnt3rep resulted in an expansion of gene expression that was dependent on Tcf-binding sites. This suggests that *HyWnt3* has the potential to be activated in a Wnt/β-catenin–dependent manner in a region broader than expected. This expansion occurs even in the context of intact HyDkk1/2/4 function. Transcriptional regulation of *HyWnt3* expression is therefore independent of HyDkk1/2/4, although HyWnt3rep action can be complemented by HyDkk1/2/4. However, a high β-catenin signal can suppress HyWnt3rep function, and the signaling center can therefore emerge by local suppression of an inhibitor being uniformly present. We speculate that the repressive activity of HyWnt3rep gets locally abolished at the onset of regeneration and budding, thereby permitting local *HyWnt3* transcription and new organizer formation. The activation of canonical Wnt signaling by alsterpaullone treatment in the presence of a putative repressor in the body column results in multiple small foci of Wnt activation (Fig. 5 *A* and *B*). Interestingly, these foci develop into tentacles, but not into complete heads (6, 36), suggesting the input of additional transcriptional activators (as detailed earlier) in head development.

Although the identity and expression pattern of the repressor molecule regulating HyWnt3rep are still unknown, it is expected to be absent from the head organizer or exist uniformly with its activity suppressed in the head organizer. HyWnt3rep contains putative binding sites for several transcription factors (Table S2). Among them, we found binding sites for the GATA transcription factor, which was reported to act as a transcriptional repressor (38). A *Hydra GATA* orthologue is expressed in the body column (Fig. S3), making it a possible repressor. Interestingly, in DNase I footprinting experiments, the *cis*-regulatory region of the *Hydra* head-specific gene *ks1* were also identified to specifically bind nuclear proteins from basal tissue (39), although the identity of the corresponding repressors is unknown. As HyWnt3prox shares 21 bp with the HyWnt3act and −2149/−386 that did not drive reporter expression in the body column, the HyWnt3prox may be a bipartite element; one represses gene expression in the lower hypostome and the other blocks expression in the body column. It is thus possible that the HyWnt3rep function is controlled by several transcriptional repressors. The identification and functional analysis of the corresponding molecules governing HyWnt3rep will clarify the mechanisms underlying the localized *HyWnt3* expression. In line with this strategy, it will be necessary to unravel the regulatory network of the cnidarian head and blastopore organizer that includes the other *Wnt* genes and yet-unidentified transcriptional repressors and activators.

The establishment of local sources of the Wnt ligand could have been important in the evolution of metazoan axis formation. We presume that the *cis*-regulatory control mechanism combining autoactivation and repression resulted in the establishment of a locally restricted Wnt signaling center. This regulatory mechanism can be independent of the function of extracellular Wnt antagonists. Although multiple Wnt antagonistic regulators have been identified from cnidarians to vertebrates, these molecules are absent from several metazoan lineages (e.g., sea urchin and some protostomes) (1). A combination of autoregulation and repression can therefore account for the localized *Wnt* expression in other animals as well. The presence of this transcriptional control in a basal metazoan suggests possible conservation in other phyla and a key role in the evolution of the spatially localized Wnt signaling centers.

Materials and Methods

Animals. The *Hydra magnipapillata* 105 strain (40) and *Hydra vulgaris* AEP strain (41) were used. Animals were cultured as described previously (42). To induce gametogenesis, the AEP animals were starved for 1 wk and then fed twice per week.

Cloning and Constructs. For the HyWnt3FL-EGFP construct, a 2,149-bp *HyWnt3* promoter fragment (HyWnt3FL) was amplified by PCR from genomic DNA of the 105 animals and subcloned into the hoT G (16), which resulted in hoT G-HyWnt3FL-EGFP (HyWnt3FL-EGFP). The derivative deletion or mutation constructs were generated by PCR and/or restriction digest (*SI Materials and Methods*), and subsequent subcloning into *Hydra* transgenesis vectors, pBSSA-AR or its derivative containing the *Hydra* hsp70 minimal promoter (pBSSA-AR-hsp.mini-EGFP; *SI Materials and Methods*), which contain the *Hydra* actin promoter and the RFP gene. Additional information on the constructs is provided in *SI Materials and Methods* and Tables S3–S7.

Generation of Transgenic *Hydra* Polyps. Generation of transgenic *Hydra* polyps was carried out as previously described (16). To ensure whether transgenic cells have a transgene, we constructed and used a vector system for *Hydra* transgenesis described in Fig. S1 and *SI Materials and Methods*. Each transgene construct was injected into 100 to 300 embryos of the AEP strain. The resultant hatched polyps were collected for 1 to 2 mo after injection and maintained individually. Polyps ubiquitously expressing the transgene were generated by clonal propagation, asexual budding. For each transgene, two to 30 independent transgenic lines were obtained, and at least two lines were analyzed. Details of generation of transgenic experiments are described in Table S3.

In Situ Hybridization. Whole-mount in situ hybridization was performed as described previously (33, 43).

Immunohistochemistry. Immunohistochemistry was performed by using GFP antibody (1:1,000; Abcam), anti-nuclear Hyβ-catenin antibody (1:100) (14), Alexa Fluor 488 goat anti-chicken IgG (1:500; Invitrogen), and Alexa Fluor 568 goat anti-guinea pig IgG (1:150; Invitrogen). DAPI staining was done with 1:5,000 dilution. *Hydra* polyps were relaxed with 2% urethane/hydra medium for 2 min, fixed with 4% formaldehyde (Sigma) for 30 min at room temperature, and then permeabilized for 30 min in PBS solution with 0.1% Triton X-100. Incubation with primary antibodies was done overnight at 4 °C in PBS with 0.1% Tween-20 with 5% donkey serum (Sigma). The anti-nuclear Hyβ-catenin antibody was produced by immunizing guinea pigs with a synthetic peptide (YQDIQRRGPGAQNMQD) encompassing amino acid region 603 to 618 of Hyβ-catenin. The antibody was affinity-purified by using the antigenic peptide.

Alsterpaullone Treatment. Animals were incubated in 5 μM alsterpaullone (Calbiochem) in *Hydra* medium for 24 h (6). Thereafter, they were rinsed with *Hydra* medium several times and cultured in *Hydra* medium.

Regeneration Experiments. Budless polyps were bisected at 80% body length.

Microscopy. Fluorescent micrographs were acquired on a Nikon 80i upright microscope equipped with a 10× PlanApo objective, NA 0.45, onto a Nikon DS-1QM cooled digital camera. Alternatively, animals were documented with a MonoZoom microscope (AZ100; Nikon) equipped with a DS-Qi1Mc cooled digital camera.

EMSA. Recombinant His-tagged XTcf-3 was expressed in *Escherichia coli* (44). EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions. Glycerol (5%), $MgCl_2$ (5mM), poly(dI-dC) (50 ng/μL), and Nonidet P-40 (0.05%) were included in the binding reaction. WT or mutant probes were present at 2 nM final concentration. The purified His-XTcf-3 was present at 63 ng/μL final concentration in the binding reaction. In competition assays, DNA oligonucleotides containing WT or mutant Tcf sites from the *X. siamois* promoter were used as competitors. Oligonucleotide sequences used in EMSA are provided in Table S4.

ChIP Assay. Approximately 180 Hyβ-catenin-EGFP transgenic animals were treated overnight with 5 μM alsterpaullone or with DMSO, and then washed with *Hydra* medium. The animals were fixed with 1% formaldehyde for 10 min and then processed using the ChIP Assay Kit (USB) according to the manufacturer's instructions. The antibody used was rabbit anti-GFP polyclonal (Abcam). The chromatin DNA was fragmented with an S-4000 sonicator (Misonix) to an average of 100 bp. The ChIP and input was compared with real time PCR by using the Chromo4 RT PCR detector attached on a DNA engine thermal cycler (Bio-Rad). Primers were designed to amplify approximately 80-bp sequences containing site 1 and sites 2 and 3, as well as a part of the HyActin promoter (sequences are provided in Table S5). The analysis was done by normalizing against input and actin promoter sequence using the $-\Delta\Delta C(t)$ method (45).

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