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Dickkopf related genes are components of the positional value gradient in Hydra

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Abstract

Hydra is a classical model organism to understand fundamental developmental biological processes such as regeneration and axis formation. Here, we show that two genes which share some similarity with members of the Dickkopf family of proteins, *HyDkk1/2/4-A* and *HyDkk1/2/4-C*, are co-expressed in gland cells and regulated by the positional value gradient. While *HyDkk1/2/4-A* is expressed throughout the gastric region, *HyDkk1/2/4-C* has a graded expression pattern with a high level of transcripts just below the tentacle zone and absence of expression in the budding zone. Blocking the activity of GSK-3β caused a drastic downregulation of *HyDkk1/2/4-C* expression in the gastric tissue. Experimental reduction of the number of *HyDkk1/2/4-C*-expressing cells resulted in expansion of the *HyWnt* expression domain in the hypostome. Thus, similar to Dickkopf proteins in vertebrates, one of the functions of *HyDkk1/2/4-C* in hydra may be to antagonize Wnt signalling.
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Introduction

Hydra represents an important invertebrate organism for evolutionary, developmental and regeneration studies (Galliot and Schmid, 2002; Bode, 2003). The single body axis is composed of a head, a body column and a foot. The tissue consists of three independent cell lineages. While the ectodermal and endodermal epithelial cells constitute the principal structural element, the interstitial stem cell system is located primarily in the ectoderm where interstitial cells are homogeneously distributed throughout the gastric region. In the hypostome and subhypostomal region as well as in the region adjacent to the basal disk, interstitial cells are absent. Interstitial cells differentiate into nematocytes, nerve cells, male and female gametes and are also capable of differentiating into gland cells (Schmidt and David, 1986; Bode et al., 1987).

The developmental processes governing the formation and maintenance of the hydra body plan are well understood at the tissue and cellular level (Bode, 2003; Bosch and Fujisawa, 2001; Steele, 2002; Bosch, 2003). The continuous cell proliferation of epithelial cells as well as interstitial cells in adult hydra results in continuous tissue displacement towards the extremities. Morphogenetic mechanisms, therefore, are continuously active in adult polyps and used for the remarkable regenerative ability, the maintenance of body shape and proportions, and the establishment of a new body axis. Crucial for axis formation is a head organizer located in the hypostome (Broun and Bode, 2002) which is thought to transmit signals to the body column (see Bode, 2003).

Numerous transplantation and tissue manipulation experiments provided experimental data for theoretical models describing the various patterning processes in hydra (Berking, 2003; Meinhardt, 1993; Meinhardt and Gierer, 2000). These models, in brief, propose that the single body axis is maintained by a morphogenetic gradient. The gradient is maximal near the head and decreases down the body column to a minimal value near the foot (Wolpert, 1971; Gierer and

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Meinhardt, 1972; MacWilliams, 1983a,b; Muller, 1996). Setting up the gradient has been proposed to involve a local autocatalytic activator that produces a long-ranging inhibitor that, in turn, antagonizes the self-activation (Meinhardt, 2004). The head forms at high levels of positional value while the foot forms at low levels.

Within the last few years, the theoretical models of axial patterning controlled by head activating and inhibiting diffusible substances have been complemented by molecular data. In particular, two sets of data point to a crucial role of Wnt genes in axial patterning. First, genes of the Wnt pathway such as *HyWnt*, *Hyβ-cat* and *HyTcf* are strongly expressed in the hypostome (Hobmayer et al., 2000). Second, blocking the activity of glycogen synthase kinase 3- beta (GSK-3β) leads to ectopic head organizer activity in the hydra body column (Broun et al., 2005). GSK-3β is an intracellular serine–threonine kinase which in the absence of a Wnt ligand targets β-catenin by phosphorylation for degradation by the proteasome (Amit et al., 2002; Liu et al., 2002).

Although these studies have identified some of the molecular mechanisms underlying hydra pattern formation, we are far from understanding the genetic program of morphogenesis. In particular, the critical molecular mechanisms that control regeneration and budding as well as the molecules which could act as carrier for positional information along the body axis are yet to be determined. To gain an overview of the transcripts controlling regeneration and budding we have performed a suppression subtractive hybridisation (SSH) analysis. This screen identified two genes belonging to the Dickkopf family, *HyDkk1/2/4-A* and *HyDkk1/2/4-C*. Both genes are coexpressed in gland cells. While *HyDkk1/2/4-A* is expressed throughout the body column, *HyDkk1/2/4-C* is expressed in a gradient like pattern with maximum expression in the subtentacle region. We provide several lines of evidence that both genes are controlled by the positional value gradient. First, we show that the expression of *HyDkk1/2/4-C* and *HyDkk1/2/4-A* is sensitive to LiCl treatment, a reagent known to affect the positional value along the body column. Second, we describe a direct influence of head and foot tissue on *HyDkk1/2/4-C* and *HyDkk1/2/4-A* expression. Third, we examine the involvement of Wnt signalling in control of *HyDkk1/2/4-C* and *HyDkk1/2/4-A* expression and show that activation of the Wnt signalling pathway by inhibition of GSK-3β inhibits expression of *HyDkk1/2/4-C*. Finally, we show that reducing the number of *HyDkk1/2/4-C*-expressing cells is correlated with increased Wnt expression in the head. We, therefore, propose that one of the functions of *HyDkk1/2/4-C* in hydra appears to be inhibition of Wnt signalling. Thus, the function of Dickkopf proteins may be conserved throughout the animal kingdom.

While this manuscript was under review, an article appeared (Guder et al., 2006) in which the authors have cloned *HyDkk1/2/4-A* by using an independent unbiased strategy. Thus, the complementary results of these two studies define an intriguing system of generating and interpreting positional value gradients in hydra in which short Dickkopf related proteins may play an essential role.

Materials and methods

Animals and culture conditions

Experiments were carried out with H*ydra magnipapillata* and *Hydra vulgaris* (Zürich strain). The animals where cultured according to standard procedures at 18°C.

Interstitial cell elimination to reduce the number of HyDkk1/2/4-C-expressing gland cells

To eliminate the interstitial cells, *H. magnipapillata* sf-1 animals raised at 18°C (permissive temperature) were incubated for 48 h at 28°C (nonpermissive temperature) and then cultured at 18°C for 3 weeks. Interstitial cells are eliminated from the treated tissue within the first 12–16 h of treatment (Marcum et al., 1989). Nematoblasts decrease within 48 h of treatment. Elimination of gland cells occurs at a slower rate and requires more than 15 days for a significant loss. Due to the loss of nerve cells and nematocytes, animals become unable to eat by themselves. These nonfeeding epithelial animals were cultured in standard medium, fed by hand, and cleaned daily using methods described (Bosch and David, 1987).

Treatment with lithium–chloride and alsterpaullone

Lithium–chloride treatment was performed as described before (Grens et al., 1996). To lower the head activation gradient along the hydra body column, 40 animals of *H. vulgaris* (Zürich strain) were incubated continuously for 14 days in 0.5 mM LiCl in hydra medium. The animals were fed every second day and washed daily. To raise the head activation gradient along the body column and to induce ectopic tentacle formation, 40 *H. vulgaris* (Zürich strain) polyps were treated with 4 mM LiCl in hydra medium for 2 days (Hassel et al., 1993). After that, they were transferred to 0.5 mM LiCl in hydra medium. The animals were fed every second day during LiCl treatment. Treatment with alsterpaullone was performed as described (Broun et al., 2005).

Molecular techniques and sequence analysis

Nucleic acid isolation, cDNA cloning, generation and characterization of the suppression subtractive hybridization (SSH) libraries, and DNA sequence analysis were carried out as described recently (Genikhovich et al., 2006). Nucleotide, protein and translated BLAST engines at the NCBI server (http://www.ncbi.nlm.gov/BLAST) were used for homology searches in public databases. The predicted amino acid sequence of *HyDkk1/2/4-A* and *HyDkk1/2/4-C* was analysed for signal peptide sequences with the help of the SignalP V3.0 (Bendtsen et al., 2004; Nielsen et al., 1997). For alignment of multiple sequences of the Dickkopf family, sequences corresponding to cysteine rich domain 2 were analysed using the Clustal W program (http://www.ebi.ac.uk/clustalw/). The following sequences were taken from the National Center for Biotechnology Information (NCBI) server: MusDkk1 (NP_034181), XenDkk1 (AAC02427), MusDkk2 (Q9QYZ8), XenDkk2 (CAC17815), HomDkk3 (Q9UBP4), GagaDkk3 (Q90839), HyDkk3 (AAQ91437), MusDkk4 (NP_663567), HyDkk1/2/4-C (DQ127904) and HyDkk1/2/4-A (DQ127903).

In situ hybridization

Methods for whole mount in situ hybridisations were adapted from previous work (Grens et al., 1996; Mitgutsch et al., 1999) and as follows: Digoxigenin- or biotin-labeled RNA probes were prepared according to the manufacturer's instructions (Roche). For double in situ hybridisation, after fixation animals were cut longitudinally to enable better probe penetration and better visualization of cells in the endoderm. Proteinase K treatment (10 μg/ml) was for 20 min at 20°C. Hybridisation was performed for 60 h at 57°C. For double in situ hybridisation, first only the biotin labelled probe was added, after 24 h also the digoxigenin labelled probe was added to the hybridisation solution. Following hybridization, unhybridised probes were removed as described by Grens et al. (1996).

Samples were incubated overnight at 4°C in 80% MAB + BSA–20% sheep serum (blocking solution) to which Alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (Boehringer-Mannheim) and, for the double in situ hybridization horseradish peroxidase-conjugated anti-biotin Fab fragments (Boehringer-Mannheim) were added. To perform the staining of the double-labeling in situ hybridization, after the NTMT washing steps the samples were washed three times 5 min with water and then two times 10 min with sodium acetate-buffer (pH 5.0). The staining reaction was carried out with 6.4 mM aminoethylcabazole (Sigma) as substrate for the horseradish peroxidase and 0.4% hydrogen peroxide in sodium acetate-buffer at 37°C for 10 to 60 min. The reaction was stopped and the substrate removed by three 10-min and one overnight washing step with water. After the red staining was documented, the second staining reaction with NBT/BCIP was carried out as above described. The double-staining was documented and thereafter the red, ethanol-soluble substrate removed and the samples dehydrated.

Results

Identification of two Dickkopf related genes expressed along the body axis

To identify genes that are differentially expressed during regeneration and budding, we performed suppression subtractive hybridisation and generated subtracted cDNA libraries enriched for cDNAs differentially expressed in regenerating and budding tissue (data not shown). One of the subtracted cDNA libraries contained a sequence cluster, which had some similarity to *HyDkk3*, the previously described hydra Dickkopf gene (Fedders et al., 2004). The 312 base pairs (bp) sequence was completed by means of the EST database, and a full-length cDNA of 528 bp containing a 5′ UTR, a start codon at position 104, an open reading frame consisting of 95 amino acids, and a 3′ UTR ending with poly-A tail was obtained (see Fig. 1A; GenBank DQ127904). Hydrophobicity analysis revealed a 19-amino acid signal peptide at the N terminus indicating that the protein may be secreted into the extracellular space. We note that whereas all members of the Dickkopf family have two cysteine rich domains, this cDNA encodes a signal peptide and a single Dickkopf-like cysteine rich domain 2 (Figs. 1A, B). We termed this gene *HyDkk1/2/4-C*. To localize *HyDkk1/2/4-C* transcripts at the cellular level, we performed in situ hybridization. *HyDkk1/2/4-C* is expressed in endodermal gland cells in a graded pattern along the body column with high expressing level in the subtentacle region (Fig. 2A). In the hypostome and subhypostomal region, as well as in the foot region, *HyDkk1/2/4-C* expression cannot be detected. Analysis of polyps in various stages of budding demonstrates that *HyDkk1/2/4-C* transcripts can be detected from intermediate budding stages on when gland cells are present (Figs. 2B–E).

Blast searches (data not shown) indicated that in addition to *HyDkk1/2/4-C* there is a second gene in the SSH library with similar structural features. The corresponding full-length sequence was obtained using the hydra EST database. The full-length cDNA of 311 bp contains a 5′ UTR, an open reading frame consisting of 93 amino acids, and a 3′ UTR (Fig. 1A; GenBank DQ127903). Hydrophobicity analysis revealed a 20 amino acid signal peptide at the N terminus. Since the predicted protein has a cysteine-rich domain, is 50% identical in amino acid sequence to *HyDkk1/2/4-C*, and is 100% identical to one of the dickkopf related genes reported in the article by Guder et al. (2006), we termed this gene *HyDkk1/2/4-A*. The amino acid alignment using the cysteine rich domain 2 shows that *HyDkk1/2/4-A* and *HyDkk1/2/4-C* are closely related to Dkk proteins in hydra, chicken, and man (Fig. 1C).

Coexpression of HyDkk1/2/4-A and HyDkk1/2/4-C in gland cells in the body column

Both genes are expressed in endodermal gland cells throughout the gastric region and are absent in the hypostome and subhypostomal region, as well as in the foot region (Fig. 2 and Fig. 3). There are two interesting differences in the expression between *HyDkk1/2/4-A* and *HyDkk1/2/4-C*. First, *HyDkk1/2/4-C* is expressed as a gradient along the body axis (Fig. 2A, Fig. 3B) while *HyDkk1/2/4-A* is uniformly expressed throughout the gastric region (Fig. 3A). The apical border of expression of both *HyDkk1/2/4-C* and *HyDkk1/2/4-A* is strikingly sharp. In contrast, the basal border of *HyDkk1/2/4-C* expression is blurred suggesting that the closer gland cells come into the vicinity of the foot the less they express *HyDkk1/2/4-C*. Second, both genes differ in their responses to signals released from regenerating tissue. In animals (n = 22) undergoing head regeneration for 2 h and 6 h, no changes in the expression of *HyDkk1/2/4-C* could be observed (Figs. 2F, G). In contrast, and in detail shown in the study by Guder et al. (Fig. 4, 2006), *HyDkk1/2/4-A* is upregulated during head regeneration (data not shown).

Since *HyDkk1/2/4-C* has a striking spatial expression pattern in the form of a gradient, and since *HyDkk1/2/4-C* and *HyDkk1/2/4-A* are closely related genes expressed in endodermal gland cells, next we asked whether the same class of gland cells coexpresses both genes. To address this question, we carried out double- in situ hybridisation experiments. As shown in Fig. 3, in the apical gastric region the same subset of gland cells expresses both genes (Figs. 3D–F). In the basal body region, the gland cells express only *HyDkk1/2/4-A*. Thus, the graded expression of *HyDkk1/2/4-C* is not due to a graded distribution of gland cells per se but due to differential gene expression in a cell population which is uniformly distributed throughout the body column. This observation points to the presence of a graded positional signal controlling *HyDkk1/2/4-C* expression in gland cells.

HyDkk1/2/4-C expression is controlled by the positional value gradient and factors released from the foot

Since *HyDkk1/2/4-C* is expressed in gland cells of the upper gastric region, but not in gland cells near the budding zone or the foot, we reasoned that examining *HyDkk1/2/4-C* expression in polyps having altered levels of positional value along the body column may reveal whether *HyDkk1/2/4-C* is inhibited in response to positional values below a specific threshold. LiCl is a reagent known to affect axial patterning and the level of the gradient throughout the hydra body column (Hassel et al., 1993). Treatment of polyps with 0.5 mM LiCl decreases the level of positional value, causing polyps to generate ectopic feet in the body column (Hassel et al., 1993; Hassel and Bieller,

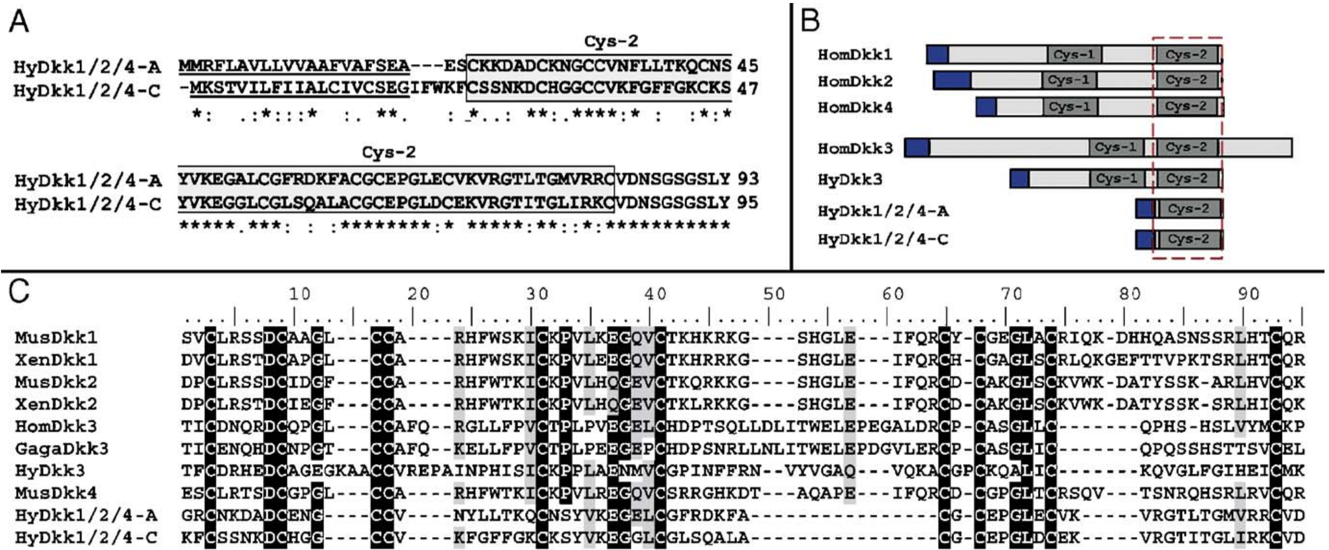

Fig. 1. *HyDkk1/2/4-C* and *HyDkk1/2/4-A* are similar in structure and related to Dickkopf proteins in vertebrates. (A) Amino-acid sequence alignment of HyDkk1/2/4-C and HyDkk1/2/4-A. *, Same amino acid residue; :, conserved substitutions; ., semi-conserved substitutions. The signal peptide sequence is underlined and the cysteine-rich domain 2 is shaded. (B) Schematic diagram depicting the structural similarities between HyDkk1/2/4-C and HyDkk1/2/4-A in comparison to HyDkk3 and Dkk-1, 2, 3 and 4 in human (HDkk). (C) Multiple amino acid sequence alignment of cystein-rich domain 2 from mouse (Mus), man (Hom), frog (Xen), chicken (Gaga) and Hydra (Hy). black boxed amino acid residues, highly conserved amino acid residues; gray boxed residues, amino acid residues sharing the same biophysical properties.

1996). As shown in Fig. 4B, 14 days after beginning of treatment, in about 80% of the polyps (31/39), the expression of *HyDkk1/2/4-C* was reduced to a region close to the apical border of the original expression domain. In contrast to *HyDkk1/2/4-C*, treatment with 0.5 mM LiCl had no influence on the expression of *HyDkk1/2/4-A* (Figs. 4C, D). The experiment shows that the graded *HyDkk1/2/4-C* expression along the body column is affected by the positional value gradient.

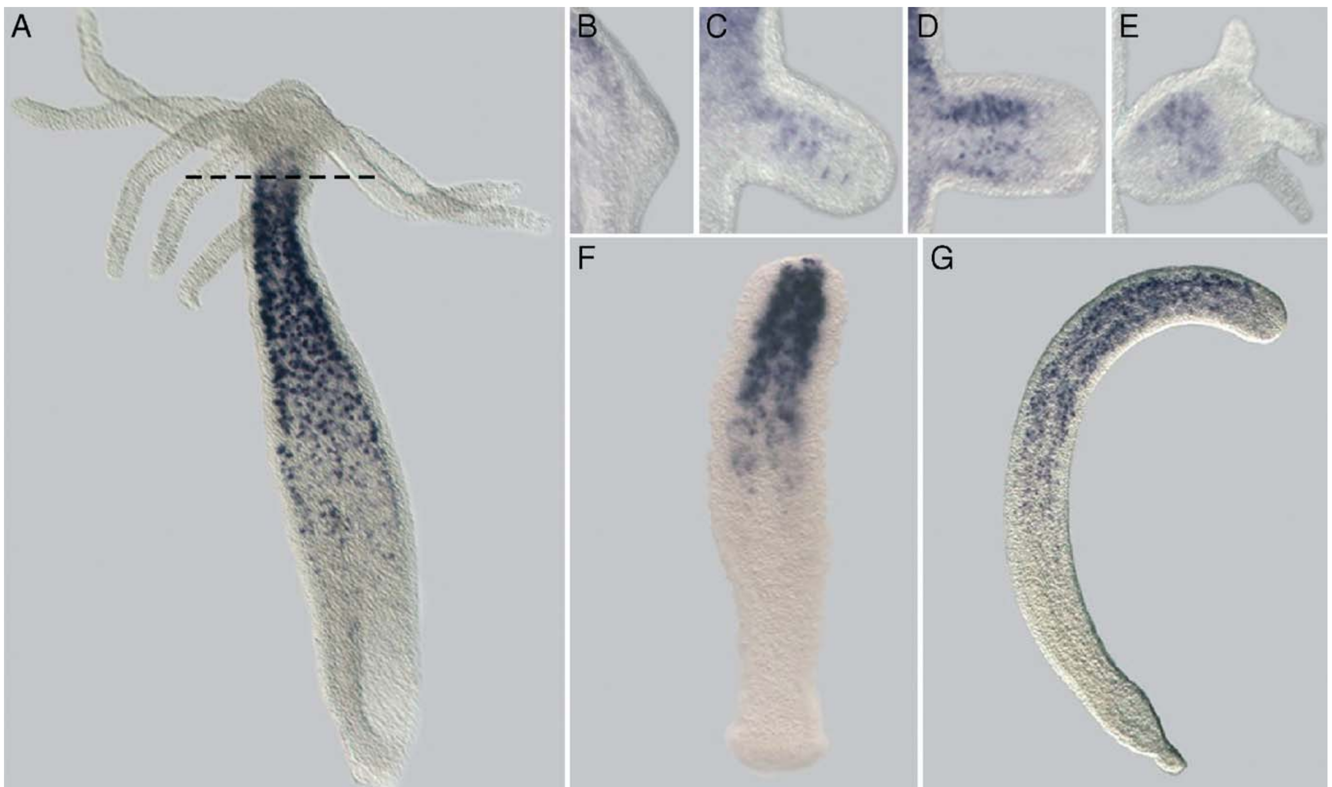

Fig. 2. Expression of *HyDkk1/2/4-C* in normal, budding, and regenerating polyps. (A) Graded expression pattern of *HyDkk1/2/4-C* along the body column. The dotted line indicates where polyps were decapitated for the regeneration experiments shown in panel F. (B–E) Expression of *HyDkk1/2/4-C* in polyps at various stages of budding. (F) Expression of *HyDkk1/2/4-C* after 2 h of head regeneration. (G) Expression of *HyDkk1/2/4-C* after 6 h of head regeneration.

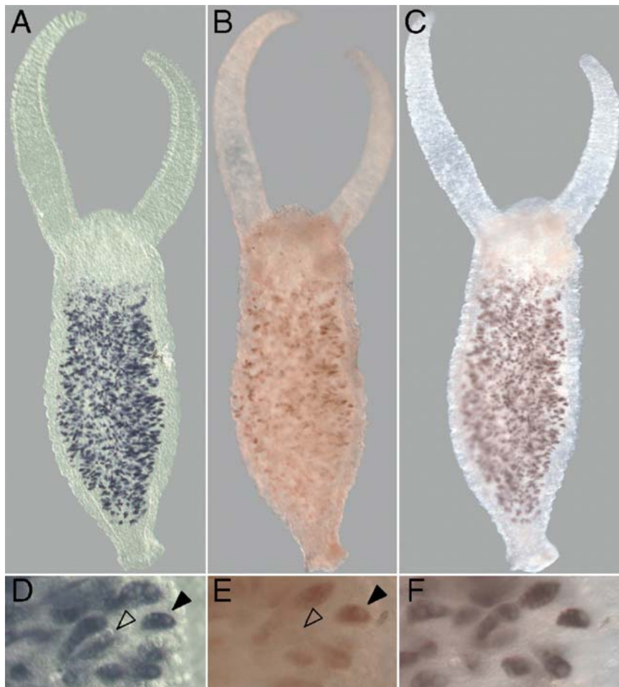

Fig. 3. *HyDkk1/2/4-A* and *HyDkk1/2/4-C* are co-expressed in endodermal gland cells in the apical part of the body as determined by double in situ hybridization. (A) *HyDkk1/2/4-A*-expressing cells in a longitudinally sectioned polyp. *HyDkk1/2/4-A* transcripts are equally distributed in gland cells along the body axis. (B) *HyDkk1/2/4-C*-expressing cells in a longitudinally sectioned polyp. Note the graded distribution of *HyDkk1/2/4-C* transcripts. (C) Double in situ hybridization reveals co-expression of both genes in the same gland cells. (D–F) Magnifications of gland cells from the polyps shown above in panels A–C. Filled arrowhead indicates a gland cell which co-expresses *HyDkk1/2/4-A* and *HyDkk1/2/4-C*; open arrowhead indicates a gland cell which expresses only *HyDkk1/2/4-A*.

To demonstrate that the foot is directly involved in regulation of *HyDkk1/2/4-C* expression, we performed two series of grafting experiments, axial and lateral grafting. First, we bisected polyps directly beneath the tentacle zone, removed the foot, and grafted the foot to the apical end of the body column (n = 23). As shown in Figs. 4G–I, due to the presence of the ectopic foot, expression of *HyDkk1/2/4-C* in gland cells is restricted to a region in the middle of the animal and absent at both ends of the graft in 86% of the grafts. A similar observation can be made in “lateral” grafts. When a foot is attached to the gastric region by “lateral” transplantation as shown in Figs. 4E–F, *HyDkk1/2/4-C* expression disappears in the vicinity of the ectopic foot in 75% (6/8) of the grafts (Fig. 4E). Both transplantation experiments demonstrate the potential of the foot to repress *HyDkk1/2/4-C* expression.

HyDkk1/2/4-C expression is transiently upregulated in tissue with high positional value

Although absent in head tissue, most gland cells expressing *HyDkk1/2/4-C* are close to the apical border near the head (Fig. 2A). Do signals from the head tissue positively influence *HyDkk1/2/4-C* expression? Prolonged treatment of animals with high concentration of LiCl raises the gradient of positional value along the body column and causes the formation of ectopic heads and individual ectopic tentacles (Hassel and Bieller, 1996). As shown in Fig. 5, two days after start of LiCl treatment ectopic head structures began to emerge in the middle of the animals (n = 79). In most cases (38 out of 42, 90%) *HyDkk1/2/4-C* expression is strongly upregulated near the emerging ectopic head (Fig. 5B). In a fully developed ectopic head (Fig. 5C), however, similar to the situation in a normal head, *HyDkk1/2/4-C* expression is inhibited in 25 out of 37 samples (67%). Thus, under these experimental conditions *HyDkk1/2/4-C* appears to be upregulated in tissue determined for head formation but not in mature heads. In contrast to *HyDkk1/2/4-C*, raising the positional value level does not increase but rather decreases the expression of *HyDkk1/2/4-A* transiently in 26 out of 37 samples (Fig. 5E). These experiments demonstrate that expression of *HyDkk1/2/4-C* and *HyDkk1/2/4-A* is controlled by factors related to axial patterning.

Activation of Wnt signalling pathway inhibits expression of HyDkk1/2/4-C

Wnt signalling involves the interaction of Wnt with the Frizzled receptor and the subsequent inhibition of glycogen synthase kinase 3-beta (GSK-3β), leading, through stabilisation of β-catenin, to transcriptional activation of target genes (Figs. 6A, B). In hydra, GSK-3β can be inhibited effectively by alsterpaullone (Broun et al., 2005). To determine whether the Wnt signalling cascade is involved in transcriptional control of *HyDkk1/2/4-C*, we, therefore, treated polyps with alsterpaullone. As expected, after 6 days of treatment, nearly all animals ($n \geq 80$) developed numerous ectopic tentacles (Fig. 6E). Strikingly, in situ hybridization revealed (Figs. 6E–F) that in these animals there is a drastic downregulation of *HyDkk1/2/4-C* expression. We note that in these animals ectopic tentacles start to appear first in the basal part of body region where gland cells do not express *HyDkk1/2/4-C* (see Fig. 6E). Since Wnt activation by alsterpaullone treatment leads to repression of *HyDkk1/2/4-C* expression in the gastric region, activation of the Wnt signalling cascade appears to antagonize *HyDkk1/2/4-C* expression.

To examine whether the gastric region of alsterpaullone treated polyps indeed has obtained “head-like” quality, we analyzed the expression pattern of *Hym-301* in alsterpaullone treated polyps ($n \geq 40$). Normally *Hym-301* is expressed in the tentacle zone and hypostome of *H. magnipapillata* (Takahashi et al., 2005). *Hym-301* is not expressed in the most apical tip of the hypostome or the tip of the bud protrusion where the *HyWnt* transcripts are localized (Hobmayer et al., 2000; Broun et al., 2005). As shown in Fig. 6G–L, after treatment with alsterpaullone *Hym-301* becomes expressed throughout the body column. Thus, as expected Wnt activation transfers head-like characteristics to the gastric column. As also shown in Fig. 6H, treatment with alsterpaullone for 6 h caused a drastic regression of *Hym-301* transcripts from the hypostome

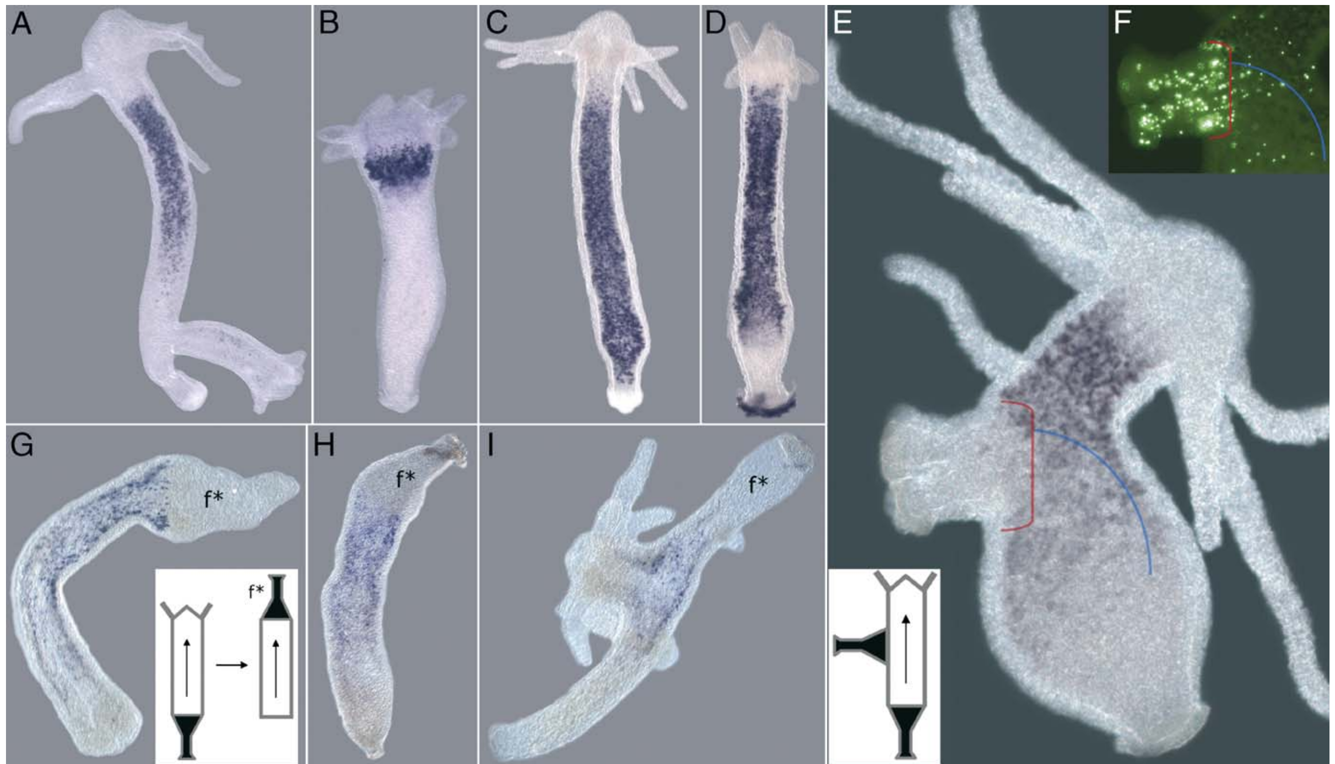

Fig. 4. *HyDkk1/2/4-C* expression is negatively controlled by factors released from the foot. (A–D) Expression of *HyDkk1/2/4-C* and *HyDkk1/2/4-A* in polyps treated for 14 days in 0.5 mM LiCl. (A and C) Control polyps. In LiCl treated polyps *HyDkk1/2/4-C* (B) is strongly repressed while *HyDkk1/2/4-A* (D) appears not to be affected. (E–F) Lateral transplantation experiments demonstrate that *HyDkk1/2/4-C* expression is negatively controlled by factor(s) released from the foot. (E) *HyDkk1/2/4-C* transcripts disappear in vicinity to the ectopic foot. The experimental procedure is shown in the inlet. (F) To demonstrate the long-range effect of the laterally transplanted foot, donor tissue was labelled by fluorescent beads. (G–I) Axial transplantation experiments demonstrate that ectopic foot tissue represses *HyDkk1/2/4-C* expression. Axial transplantation of foot (f *) followed by in situ hybridisation using a *HyDkk1/2/4-C* specific probe at 0 days (G), 2 days (H) and 6 days (I) after transplantation. The experimental procedure is depicted in G.

expression domain. Interestingly, Broun et al. (2005) have reported previously that treatment of hydra with alsterpaullone causes an expansion of the *HyWnt* domain from the apical tip of the hypostome to cover a larger area of the hypostome. Thus, the data shown in Figs. 6C–F indicate that activation of the Wnt signalling cascade appears to antagonize *HyDkk1/2/4-C* expression.

In vertebrates, Dickkopf proteins are antagonists of Wnt signalling. To examine whether in hydra *HyDkk1/2/4-C* also antagonizes Wnt signalling, we generated tissue which had the number of *HyDkk1/2/4-C*-expressing gland cells reduced by about 75% (see Supplementary Fig. 1) compared to controls by using *H. magnipapillata* mutant strain sf1 polyps. Fig. 6N indicates that – similar to the effects of alsterpaullone on Wnt expression reported by Broun et al. (2005) – in tissue with a reduced number of *HyDkk1/2/4-C*-expressing cells, the *HyWnt* expression domain is expanded. Moreover, the depletion of *HyDkk1/2/4-C* causes a drastic regression of both the *Hym301* and *ks1* expression domains in the hypostome (Figs. 6P and R). Normally, in control polyps (Figs. 6O, Q; see also Endl et al., 1999; Takahashi et al., 2005) these effector genes for head formation are expressed throughout the hypostome but not in the most apical tip of the hypostome where *HyWnt* transcripts are localized. Thus, removal of *HyDkk1/2/4-C*-expressing cells appears to have the same effects as activation of the Wnt signalling cascade by alsterpaullone treatment. Based on these data, we conclude that similar to Dickkopf in higher animals, one of the functions of *HyDkk1/2/4-C* may be to antagonize Wnt signalling.

Discussion

Regeneration and mechanisms controlling axis formation in hydra are both complex and dynamic. They require positional information, involve self-regulation, and lead to the establishment of polarity and position dependent differentiation. Here, we provide evidence that Dickkopf related proteins are elements of the positional value system in hydra.

Dickkopf related genes in hydra

Our screen for transcripts essential for tissue remodelling/regeneration in hydra resulted in the identification of two genes with similarity to members of the Dickkopf family. In both genes, *HyDkk1/2/4-A* and *HyDkk1/2/4-C*, only a Dickkopf-like cysteine rich domain 2 is present. Both *HyDkk1/2/4-A* and *HyDkk1/2/4-C* are structurally very similar (Fig. 1) and are produced in endodermal gland cells. Although this cell type is uniformly distributed along the

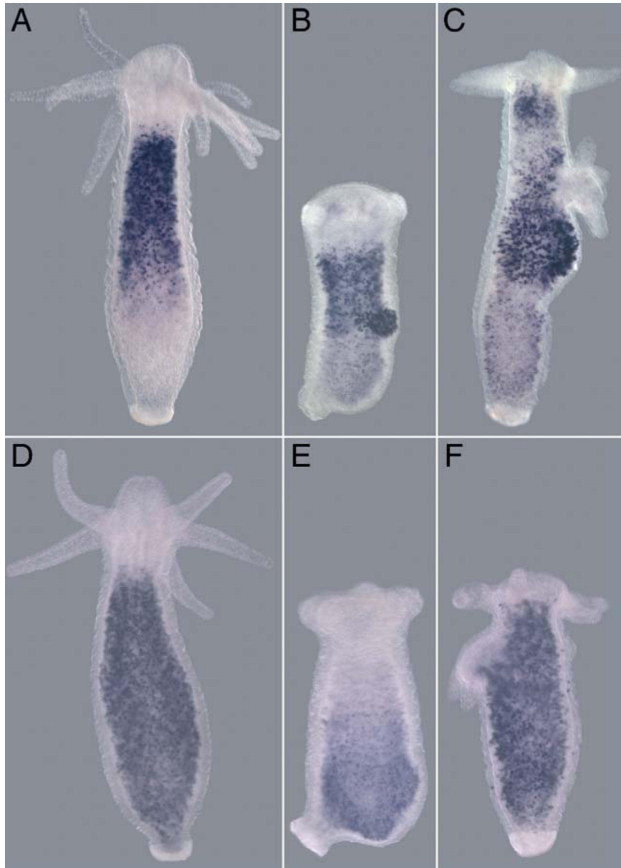

Fig. 5. *HyDkk1/2/4-C* expression is upregulated in tissue with high positional value. (A–C) *HyDkk1/2/4-C* expression in polyps treated with LiCl at a concentration and time regime which raises the positional value along the body column (Hassel and Bieller, 1996). (D–F) *HyDkk1/2/4-A* expression is downregulated by increasing the positional value; (B and E) expression after two days of LiCl treatment; (C and F) expression after 8 days of LiCl treatment.

body column (Fig. 3), both genes have different expression patterns (Fig. 3) and respond to positional signals in a different manner (Fig. 4 and Fig. 5). We have reported previously that hydra has one and most likely only one bona fide member of the Dickkopf family, *HyDkk3*, which contains both cysteine rich domains typical for Dickkopf proteins in vertebrates (Fedders et al., 2004). This fact led us to propose that the four vertebrate *Dkk* genes might have originated from a common ancestor gene related to *HyDkk3* (Fedders et al., 2004). The identification of *HyDkk1/2/4-A* and *HyDkk1/2/4-C* in the present study raises the important question whether these small proteins can be considered as structural and/or functional precursors of the conventional Dkk molecules.

HyDkk1/2/4-C and the positional information system along the hydra body column

In vertebrates, members of the Dickkopf family have essential roles during embryogenesis and adult tissue maintenance. Perturbations in Dickkopf function promote both human degenerative diseases and cancer (Logan and Nusse, 2004). Dickkopf proteins have been identified as inhibitors of Wnt signalling by binding to the LRP5/LRP6 component of the Wnt receptor complex (Kawano and Kypta, 2003). Can this function be traced back to the origin of metazoan evolution? Two lines of evidence presented here show that *HyDkk1/2/4-C* in hydra is involved in position dependent cell-signalling along the body axis. First, along the oral–aboral axis *HyDkk1/2/4-C* is expressed in a gradient-like pattern (Fig. 2A and Fig. 3). Since expression of the gene is continuously down-regulated in cells which leave the gastric column and enter head or foot tissue, expression of *HyDkk1/2/4-C* is connected to, or regulated by the positional value gradient. Second, treatments with reagents such as LiCl or alsterpaullone as well as transplantation experiments demonstrate that expression of *HyDkk1/2/4-C* is dependent on the positional value gradient. Interestingly, although both LiCl and alsterpaullone treatment increase the positional value, their influence on the expression of *HyDkk1/2/4-C* is different: in alsterpaullone treated polyps (see Fig. 6) *HyDkk1/2/4-C* expression is repressed throughout the whole gastric column, whereas in LiCl treated animals *HyDkk1/2/4-C* expression is activated in regions where ectopic heads will be formed (Fig. 5B). We assume that the different influence on the expression of *HyDkk1/2/4-C* reflects different functions of the two compounds. While alsterpaullone is capable to transform the whole gastric region into head like tissue, LiCl is not only able to transform the gastric tissue into head like tissue but in addition can induce ectopic heads. In Fig. 5C, we show that in LiCl treated polyps an ectopic head is formed right next to *HyDkk1/2/4-C*-expressing cells. The newly formed head contains no *HyDkk1/2/4-C*-expressing cells—similar to gastric tissue treated with alsterpaulone (see Fig. 6F). Thus, although both alsterpaulone as well as LiCl increase the positional value, they seem to function in a different way.

Reducing the number of *HyDkk1/2/4-C*-expressing cells leads to an increase in the expression domain of *Wnt* in the hypostome (Fig. 6N). Enlargement of the *Wnt* expression domain is correlated with disappearance of *ks1* and *Hym301* transcripts from the upper part of the hypostome (Figs. 6P and R) indicating that both genes may be controlled by the Wnt pathway. Interestingly, a recent report by Broun et al. (2005) shows that blocking the activity of GSK-3β by alsterpaullone also leads to an expanded *HyWnt* expression domain covering a large area of the hypostome. Thus, reducing the number of *HyDkk1/2/4-C*-expressing cells seems to have the same effect as activation of Wnt. This may indicate that – similar to Dickkopf proteins in vertebrates – *HyDkk1/2/4-C* in hydra may act as extracellular inhibitor of the Wnt pathway. In this context it is interesting to note that in mammalian Dkk-1 and Dkk-2 the Wnt inhibiting function is localized in the second cysteine rich domain (Li et al., 2002; Kawano and Kypta, 2003). Since in hydra activation of *HyWnt* by blocking the activity of GSK-3β drastically represses *HyDkk1/2/4-C* expression (Fig. 6), not only *HyDkk1/2/4-C* appears to interact negatively with Wnt but

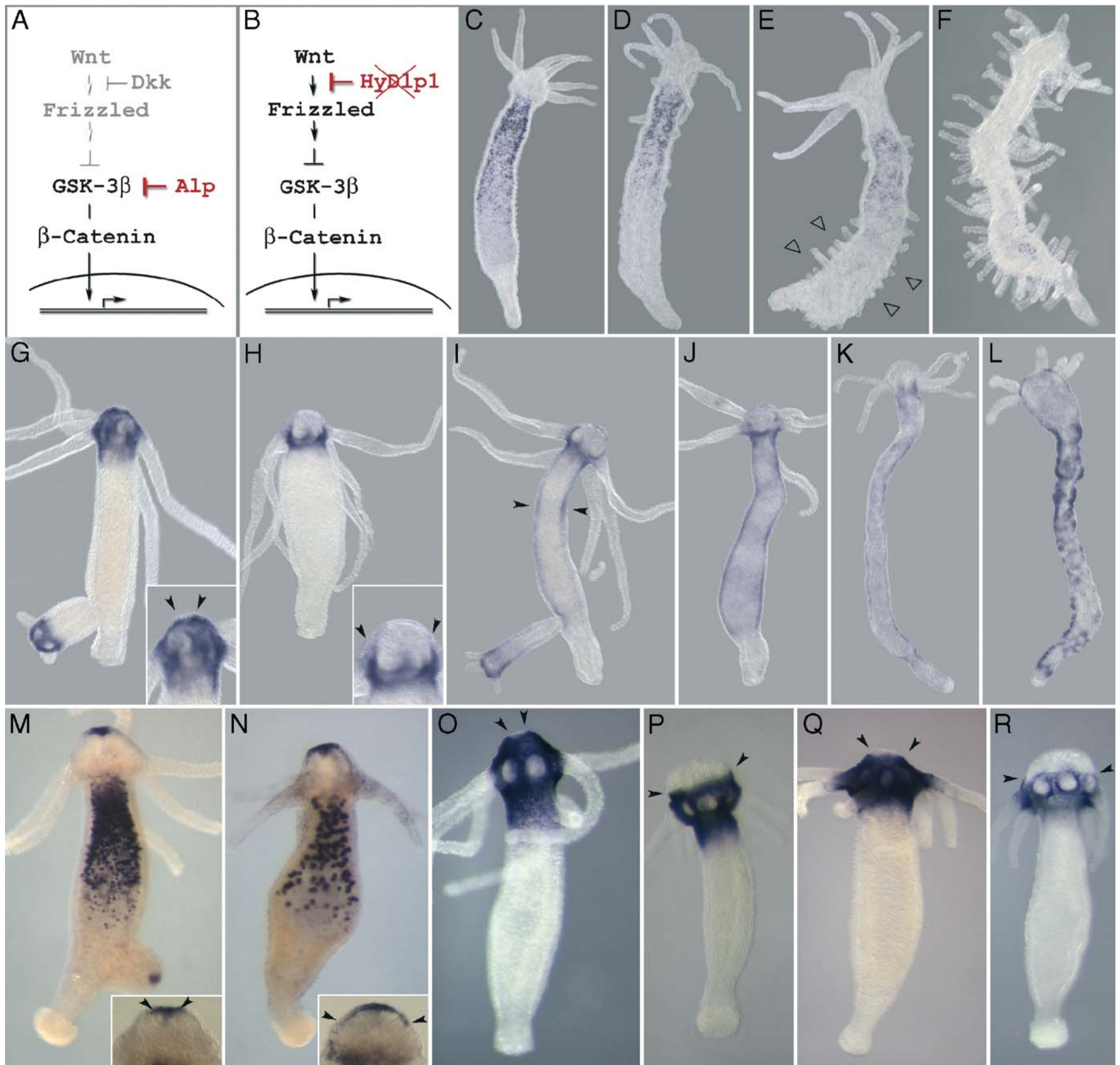

Fig. 6. (A and B) Scheme of experiments performed to interfere with Wnt signalling in hydra. (C–F) Blocking the activity of GSK-3β by alsterpaullone leads to inhibition of *HyDkk1/2/4-C* expression. Days after beginning of treatment: (C), 2 days; (D) 4 days, (E); 6 days, (F) 9 days. Arrowheads indicate that ectopic tentacles first appear in a region where no *HyDkk1/2/4-C* transcripts can be detected. (G–L) Blocking the activity of GSK-3β by alsterpaullone leads to disappearance of *Hym301* transcripts from the hypostome and to induction of *Hym301* expression along the body column. Hours after beginning of treatment: (G) 3 h; (H) 6 h, (I) 12 h; (J) 24 h; (K) 48 h; (L) 72 h. Arrowheads indicate that expression of Hym301 in treated polyps is reduced in the hypostome and induced along the body column. (M–N) Reducing the number of *HyDkk1/2/4-C*-expressing cells (see also supplementary Fig. 1) leads to expanded expression domain of *HyWnt* in the hypostome compared to control polyp. Double in situ hybridisation with *HyDkk1/2/4-C* and *HyWnt*. Arrowheads in inlets indicate the expression domain of *HyWnt*. (O and P) Disappearance of *Hym301* transcripts in the hypostome of a polyp with a reduced number of *HyDkk1/2/4-C*-expressing cells. Control polyp is shown in panel O. (Q and R) Disappearance of *ks1* transcripts in the hypostome of a polyp with a reduced number of *HyDkk1/2/4-C*-expressing cells. Control polyp is shown in panel Q.

Wnt itself seems to be involved in control of *HyDkk1/2/4-C* expression. Based on the observations we propose that *HyDkk1/2/4-C* protein in hydra confines Wnt activation to the apical tip of the hypostome or the evaginating bud. Moreover, since buds only develop in a body region where the *HyDkk1/2/4-C* level is low or absent (Fig. 2B), *HyDkk1/2/4-C* may act as repressor of bud formation in the upper part of the body column.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.003.

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