Development 133, 901-911 doi:10.1242/dev.02265

An ancient Wnt-Dickkopf antagonism in *Hydra*

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The *dickkopf* (*dkk*) gene family encodes secreted antagonists of Wnt signalling proteins, which have important functions in the control of cell fate, proliferation, and cell polarity during development. Here, we report the isolation, from a regeneration-specific signal peptide screen, of a novel *dickkopf* gene from the fresh water cnidarian *Hydra*. Comparative sequence analysis demonstrates that the Wnt antagonistic subfamily Dkk1/Dkk2/Dkk4 and the non-modulating subfamily Dkk3 separated prior to the divergence of cnidarians and bilaterians. In steady-state *Hydra*, *hydkk1/2/4*-expression is inversely related to that of *hywnt3a. hydkk1/2/4* is an early injury and regeneration responsive gene, and *hydkk1/2/4*-expressing gland cells are essential for head regeneration in *Hydra*, although once the head has regenerated they are excluded from it. Activation of Wnt/β-Catenin signalling leads to the complete downregulation of *hydkk1/2/4* transcripts. When overexpressed in *Xenopus*, HyDkk1/2/4 has similar Wnt-antagonizing activity to the *Xenopus* gene Dkk1. Based on the corresponding expression patterns of *hydkk1/2/4* and neuronal genes, we suggest that the body column of *Hydra* is a neurogenic environment suppressing Wnt signalling and facilitating neurogenesis.

KEY WORDS: Dickkopf, *dkk*, Wnt signalling, Wnt antagonism, Regeneration, Axis formation, Neurogenesis, Cnidaria, *Hydra*, *Xenopus*

INTRODUCTION

Secreted Wnt glycoproteins constitute one of the major families of cell signalling molecules (Logan and Nusse, 2004; Nelson and Nusse, 2004; Nusse, 2003), and their appearance in early metazoan evolution was probably linked to the origin and evolution of multicellular animals (Kusserow et al., 2005). In vertebrate embryonic development, Wnt ligands initiate signalling by interacting with two types of receptor molecules: the seven-pass trans-membrane protein Frizzled and the low-density lipoprotein receptor Lrp5/Lrp6. It is currently assumed that Wnt ligands bind to both Lrp5/Lrp6 and Frizzled to form a functional ligand dual-receptor complex that activates the canonical Wnt-β-Catenin pathway. Dickkopf 1 (Dkk1), a major secreted Wnt antagonist, binds to Lrp5/Lrp6 and its co-receptor Kremen to inhibit Wnt signalling (Davidson et al., 2002; Mao et al., 2002; Mao et al., 2001; Semenov et al., 2001). Inhibition of canonical Wnt signalling by Dkk1 is essential for head formation and limb patterning in the vertebrate embryo (Davidson et al., 2002; Glinka et al., 1998; Grotewold and Ruther, 2002b; Hashimoto et al., 2000; Kazanskaya et al., 2000; Mukhopadhyay et al., 2001; Shinya et al., 2000).

Of the four Dickkopf (Dkk) protein family members, Dkk1, Dkk2 and Dkk4 are able to inhibit Wnt signalling (Glinka et al., 1998; Krupnik et al., 1999; Mao and Niehrs, 2003; Wu et al., 2000). In addition, Dkk2 can act as an Lrp6 agonist, as well as antagonist, depending on the cellular context (Mao and Niehrs, 2003; Wu et al., 2000). Dkk3 is unique within the Dickkopf family in that it is not able to antagonize Wnt signalling (Krupnik et al., 1999; Mao and Niehrs, 2003).

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Accepted 28 December 2005

Vertebrate Dickkopf molecules consist of two cysteine-rich domains (CRD1 and CRD2), which are separated by a spacer region, diagnostic for grouping of Dkk proteins. It has been shown that CRD2 is necessary and sufficient to repress canonical Wnt signalling by competing with the Wnt-Frizzled complex for binding to the Lrp5/Lrp6 receptor. CRD1 is thought to have a modulating function on CRD2 (Brott and Sokol, 2002; Li et al., 2002).

Hitherto, it is completely unknown at what point in metazoan evolution Wnt-Dickkopf antagonism was established. Although the receptor proteins Frizzled and Lrp5/Lrp6 from vertebrates have homologues in insects, no Dickkopf protein could be identified in the insect and nematode genomes. A Dkk3-related protein was recently identified in *Hydra* (Fedders et al., 2004), and it was suggested that Dkk3 represents the ancestral Dickkopf type. It was furthermore proposed that subsequent genome duplication created the vertebrate Dkk1/2/4 subfamily (Fedders et al., 2004). According to this proposal, the Dickkopf-Wnt antagonism was established relatively late in metazoan evolution, i.e. concomitant with the radiation of vertebrates.

We describe here a new Dickkopf-related molecule from the freshwater polyp *Hydra* and the starlet sea anemone *Nematostella*. The novel *Hydra* Dickkopf protein was isolated as regeneration specific in a yeast signal peptide secretion screen. Structural and phylogenetic analysis indicates that this *dkk* gene is a *dkk1/2/4* homologue, from which the vertebrate *Dkk1*, *Dkk2* and *Dkk4* arose by gene duplication. Moreover, our expression and functional analyses support the view that the Wnt-Dickkopf antagonism is phylogenetically very old, and probably had a major function in setting up a neurogenic environment in the body column of the pre-bilaterian *Hydra*.

MATERIALS AND METHODS

Hydra culture and experiments

Hydra strains *Hydra magnipapillata* 105 and sf-1 (Sugiyama and Fujisawa, 1978), as well as *Hydra vulgaris* Basel and AEP (Martin et al., 1997), were cultured at 18°C (Takano and Sugiyama, 1983), fed five times a week, and used 24 hours after feeding for experiments. Gametogenesis was induced in polyps of *Hydra vulgaris* strain AEP as described (Rentzsch et al., 2005).

Elimination of interstitial cell lineage

Temperature-sensitive interstitial cells of *Hydra magnipapillata* strain sf-1 were eliminated by culturing polyps at 28-30°C for 5 days; afterwards starved polyps were kept at 18°C for up to six weeks (see Results). Loss of interstitial cells and derivatives was examined in macerates (David, 1973).

Regeneration and wounding experiments

Polyps were bisected at 20%, 50% or 80% body length by using a small scalpel. In some experiments, heads were removed without injury by tying a knot with a thin hair around the subhypostomal region of the polyp as described (Newman, 1974). This caused the head to be gradually pinched off without leaving an open wound with exposed endoderm. For wounding, one deep cut was set into the middle of the body column (30% body width). All animals were transferred into fresh medium after treatment.

Alsterpaullone treatment

Alsterpaullone (Calbiochem) was dissolved in DMSO and diluted with hydra medium to 5 μM. Daily fed polyps were incubated in alsterpaullone for 24 or 48 hours.

Molecular techniques

Isolation of mRNA

We isolated polyA+ RNA from total RNA (Suzuki et al., 2001) of *Hydra vulgaris* strain Basel, *Hydra magnipapillata* strain sf-1 (nf-1), and *Hydra magnipapillata* strain 105, by using the PolyATtract mRNA Isolation System III (Promega).

Cloning of *hydkk1/2/4*

A *hydkk1/2/4* cDNA clone was isolated in a screen for organizer-specific transcripts from the i-cell depleted *Hydra magnipapillata* strain sf-1 by using the signal peptide secretion approach (Fig. 1) (Jacobs et al., 1997; Jacobs et al., 1999). Blast search revealed a match with clone taa05h01 (CA303262) from the hydra EST collection (http://mpc.uci.edu/hampson/public_html/blastlif9). This sequence contains a complete polyA tail. The full-length sequence was amplified from *Hydra magnipapillata* strain 105 cDNA by 5′RACE-PCR using the GeneRacer Kit (Invitrogen), and specific primers (5′-CCGCAGAGTGCACCT-TCTTTAACATAGCTATTACATTGC-3′ and 5′-GCAGTCTGCATCCT-TTTTGCAAGACTCGGC-3′). Complete *hydkk1/2/4* cDNAs were additionally amplified from both, *Hydra vulgaris* strain Basel and *Hydra magnipapillata* strain sf-1 by using oligo-dT primed cDNA and specific primers (5′-GAAAACATACATCTTTTCTGATTTATCAATC-3′ and 5′-(T)17ATAATTTAACTCG-3′). PCR amplicons were cloned into the pGEM-T vector (Promega). A sequence comparison of the three strains revealed that *hydkk1/2/4* from *Hydra magnipapillata* sf-1 exhibited 10 nucleotide substitutions within the ORF, resulting in seven amino acid exchanges. For all experiments the *Hydra vulgaris* clone was used.

Cell culture experiments

TOPFLASH assay in HEK293T cells was performed as previously described (Wu et al., 2000). Transformation of cells was carried out in 96-well plates, in triplicate, with 1 ng each of *GFP*, *renilla* and *hfrizzled1*, 10 ng TOPFLASH vector, 8 ng *mwnt1*, 3 ng human *LRP6*, 20 ng *hydkk1/2/4*, 5 ng *xdkk1* and 3.125 ng *hkrm1*. Samples were supplemented with $pCS2^{+}$ vector to 100 ng DNA. After 24 hours, the transformation efficiency was checked by monitoring GFP fluorescence. Firefly luciferase activity was normalized against renilla.

In situ hybridization (ISH)

Whole-mount ISH was performed as described (Grens et al., 1996; Martinez et al., 1997) at a probe concentration of 0.05 ng/μl for 36 hours. Double ISH was performed as described (Hansen et al., 2000; Philipp et al., 2004), with NBT/BCIP substrate followed by Fast Red substrate. Sense and antisense riboprobes were produced from a 534 bp fragment of *hywnt3a* (bp 624-1158) and from full-length *hydkk1/2/4* using the DIG or FITC RNA Labelling Kit (Roche).

Macerate ISH was performed by preparing a fixed cell suspension of macerated cells (David, 1973) dropped (100 μl) onto a poly-L-Lysine-covered object slide and allowed to dry for 45 minutes at room temperature. After washing for 5 minutes with PBS/PBS_{Tween}, preparations were pre-hybridized (2 hours, 60°C), hybridized (24 hours, 60°C, 0.1 ng/μl probe) and washed (2×SSC, 2×5 minutes, 60°C; 2×SSC+0.1 % CHAPS at 50°C and 40°C), then sequentially incubated with blocking reagent (3 hours at room temperature) and anti-digoxigenin monoclonal antibody (4000-fold diluted, overnight 4°C). Detection was carried out as described for whole-mount ISH.

In vitro transcription of *hydkk1/2/4* and *hywnt3a* mRNA

The complete *hydkk1/2/4* ORF was amplified and cloned as a *Bam*HI/*Cla*I fragment using the oligonucleotides 5′-CTTTTCGGATCCATCAAT-3′ and 5′-GTATTTAAATCGATACAAAGATCCAC-3′ into pCS2+ and pCS2+MT (myc-tag) expression vectors (Rupp et al., 1994). The *hywnt3a* ORF was amplified from *Hydra vulgaris* cDNA as a *Cla*I/*Xba*I fragment using the oligonucleotides 5′-CATCGATTTGCCGCCACCATGGGCACG-3′, including the KOZAK sequence, and 5′-TTTCTAGACTATTTACAGG-TGTATTCAG-3′, and cloned into pCS2+ vector. PCR conditions for all amplicons were: 2 minutes at 94°C, then 30 cycles of 30 seconds at 94°C, 30 seconds at annealing temperature and 1 minute at 72°C; the annealing temperature was 55°C for the complete *hydkk1/2/4* sequence, 46°C for the *hydkk1/2/4* ORF and 52°C for the *hywnt3a* ORF. Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) or by the GATC Company (Konstanz).

Phylogenetic analyses

Protein sequence alignment was performed using the TCoffee alignment tool (http://igs-server.cnrs-mrs.fr/Tcoffee) and adjusted manually. Maximum likelihood trees were computed using IQPNNI (Vinh and von Haeseler, 2004) (300 repetitions) and quartet puzzling as implemented in TREE-PUZZLE 5.2 (Schmidt et al., 2001) (10,000 intermediate trees), both using the VAG model of evolution.

The following sequences were used: HyDkk1/2/4-A; HyDkk1/2/4-B (EST CN559480); *Nematostella vectensis* Dkk1/2/4, Dkk3 (contigs 7341 and 9370); *Acropora millepora* (EST GS01bF09.b1); *Branchiostoma belcheri tsingtaunese* (EST AY608670); Zebrafish (*Danio rerio*) Dkk1 (BAA82135); *Xenopus* Dkk1, Dkk2 (AAC02427, XLA300197); mouse Dkk1, Dkk2, Dkk3, Dkk4 (NM_015789, NM_020265, NM_015814, NM_145592); human Dkk1, Dkk2, Dkk3, Dkk4 (BAA34651, BAA85465, BAA85488, BAA33475); *Dictyostelium discioideum* WGS_BC5V2_0 (Sanger Institute); human and mouse Colipase (Col) (AAP35458, AAL40731).

Microscopy

Specimens were analyzed using a Zeiss Stemi SV 11 binocular, a Zeiss Axiovert 100, or a Nikon Eclipse-80i microcope, both equipped with interference contrast. For microphotography, either a Diagnostic Instruments Spot-II, a Canon PowerShot G5 or a Nikon DSL-1 camera were used. Micrographs were processed using the manufacturer's software in combination with MetaMorph or Adobe Photoshop software.

Xenopus experiments

In vitro fertilization, embryo culture, staging, microinjection and culture of explants were carried out as described (Gawantka et al., 1995). mRNA was produced with the MessageMachine SP6 Kit (Ambion) from the *hydkk1/2/4* ORF and the *hywnt3a* ORF in the *Not*I linearized pCS2+ vector; mRNA was purified with P6 Spin Columns (Bio-Rad). Injections were done radially into all blastomeres of four-cell-stage *Xenopus laevis* embryos (100 pg), or in case of *hywnt3a* radially into ventral blastomeres (1 ng). Control mRNA (10 pg *xdkk1*, 12.5 pg *xwnt8*) was injected as well.

Animal cap assay

Xenopus laevis embryos were injected in the animal pole of all blastomeres at the four-cell stage: *xwnt8* (100 pg), *xdkk1* (200 pg), *hydkk1/2/4* (6 ng). Animal caps were explanted at stage 8-9 and analyzed by RT-PCR at stage 10 for the induction of *siamois* expression. *xbra* expression validates mesoderm-free caps, *histone-4* expression was monitored for normalization.

Luciferase assays

Xenopus laevis embryos were injected in all blastomeres at the four-cell stage, and subequatorially with the Wnt-reporter construct siamois-luciferase p01234 alone or in conjunction with *xwnt8* (150 pg), *xdkk1* (300

pg) and *hydkk1/2/4* (750 pg and 3 ng). Embryos were collected at stage 10-10.5 in Passive Lysis Buffer (Promega) (25 μl/embryo). Results are presented as Relative Luciferase Units (RLU).

Secondary axes assay

Xenopus laevis embryos were injected into two opposite blastomeres at the four-cell stage with *wnt8* (12.5 pg), *xdkk1* (10 pg) and *hydkk1/2/4* (1 ng).

RESULTS

Identification of a Dickkopf-related molecule from *Hydra* in a yeast signal peptide secretion screen

In order to identify growth factors and their antagonists in *Hydra*, we performed a signal peptide secretion screen (Jacobs et al., 1999; Klein et al., 1996). Because Marcum and Campbell (Marcum and Campbell, 1978) have shown that *Hydra* lacking nerve cells, nematocytes and interstitial cells develop normally, we tried to eliminate the highly abundant transcripts of this cell line. We used heat-shocked animals of the mutant strain sf-1 from *Hydra magnipapillata*, which had lost their interstitial stem cells and nematocytes. After inducing head regeneration, we collected the heads and isolated regenerating stumps at various time-points after head removal (0.5 to 24 hours), and prepared a regeneration-specific cDNA library (Fig. 1A). The most abundant clone (10 %) in the signal peptide secretion screen contained a 328 bp fragment encoding a cysteine-rich protein. Using 5′RACE we completed the sequence obtaining a 395 bp transcript with short 5′ and 3′UTRs (88 bp and 31 bp, respectively). The open reading frame encodes a protein of 73 amino acids, containing a cysteine-rich motif and a signal peptide of 19 amino acids (see Fig. S1A in the supplementary material). BLAST searches at NBCI revealed with highest score (E-value 0.005) similarity to the CRD2 of vertebrate Dickkopf subfamily members Dkk1, Dkk2 and Dkk4. The CRD2 motif of the Dkk family shares a high structural similarity with colipases (coenzymes of lipases) and has been assigned to the colipase fold (Aravind and Koonin, 1998). However, the similarity of the novel *Hydra* Dkk protein to colipases was much lower (E-value 0.62).

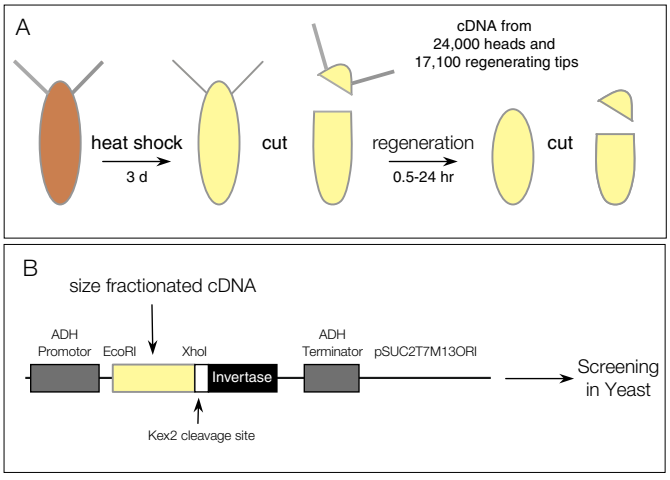

Fig. 1. Isolation of secreted molecules from the *Hydra* head organizer in a yeast signal peptide secretion screen. (**A**) Polyps of the temperature-sensitive strain *Hydra magnipapillata* sf-1 were exposed to heat shock for 3 days, causing the elimination of interstitial cells. Heads, as well as regenerating tips, were isolated from the heat-shocked polyps at various times after head removal for mRNA and cDNA synthesis. (**B**) Cloning strategy. Size-fractionated cDNA was cloned adjacent to a signal peptide-deficient yeast *invertase* gene for expression in the yeast strain YTK12. Clones expressing a fusion protein with an intact *Hydra* signal peptide were selected by growth on raffinose plates.

Phylogenetic analysis of the Dickkopf-related molecule reveals features of a putative Dkk1/2/4 precursor

Another Dkk-related molecule was recently identified in *Hydra*, which shares structural features with the vertebrate Dkk3 and was classified as a Dkk3 orthologue (Fedders et al., 2004). Because the novel *Hydra* Dkk-related molecule identified in the screen shows a higher similarity to CRD2 of the vertebrate Dkk1/2/4 subfamilies, we hypothesized that it might be a new member of the Dkk family in cnidarians. For a better overview, we performed a search for members of the Dkk family using EST data from several other invertebrates. We found ESTs from two other cnidarians, *Nematostella vectensis* and *Acropora millepora*, from the urochordate *Branchiostoma belcheri tsingtaunese*, and from the cellular slime mold *Dictyostelium.* No Dkk-like proteins were found in insect and nematode genomes.

The *Nematostella* contigs encode for two different Dkk-like proteins, each comprising two complete cysteine-rich domains (see Fig. S1A,B in the supplementary material). Alignments using TCoffee (Fig. 2A), but also ClustalW and Muscle (data not shown), reveal a higher similarity of cnidarian Dkks to vertebrate Dkks than to vertebrate colipases. In cnidarian Dkks, all ten cysteine residues are completely conserved, and some other amino acids, like glycines and lysines, are partially conserved among the species. Vertebrate Dkk1, Dkk2, and Dkk4 share a number of motifs that cannot be found in the vertebrate Dkk3 subfamily. Cnidarian Dkks share a number of amino acids with both the Dkk1/2/4 and the Dkk3 subfamilies, but again not with the colipases (Fig. 2A).

The IGPNNI ML-tree (Fig. 2C) of the CRD2 shows two distinct cnidarian Dkk subtrees: one containing the more *dkk3*-like sequences, the other containing the *Hydra* Dkk-related protein isolated in the secretion screen and the second *nvdkk*. The latter subtree is positioned closer to the highly supported cluster of the vertebrate Dkk subfamilies 1, 2 and 4. Unfortunately, neither TREE-PUZZLE nor bootstrap analyses were able to resolve any of the inner branches due to the high divergence of the Dkk family. However, a basal position between the Dkk3 and the Dkk 1/2/4 subfamilies was never contradicted. For clarity, we refer to the novel cnidarian Dkks as NvDkk1/2/4 and HyDkk1/2/4 (see Discussion).

hydkk1/2/4 is expressed in endodermal derivatives of the interstitial stem cell lineage

In order to unravel the function of the putative *hydkk1/2/4* gene, we analyzed its expression pattern by in situ hybridization. Strikingly, *Hydra*'s head was completely free of *hydkk1/2/4*-expressing cells, whereas the entire body exhibited a strong endodermal expression with a sharp boundary below the tentacle formation zone (Fig. 3A,B) (see also Hobmayer et al., 1990a; Hobmayer et al., 1990b). In many specimens, we found a graded expression, diminishing towards the peduncle region. This expression pattern is inversely related to that of *hywnt3a*, *hytcf*, *brachyury* and other head-specific genes (Hobmayer et al., 2000; Technau and Bode, 1999).

hydkk1/2/4 is not expressed in endodermal epithelial cells, but is present in gland cells. Gland cells are derivatives of the interstitial stem cell lineage (i-cells), which also gives rise to nerve cells, nematocytes and sex cells. To verify the i-cell nature of *hydkk1/2/4*-expressing gland cells, we investigated the *hydkk1/2/4*-expression

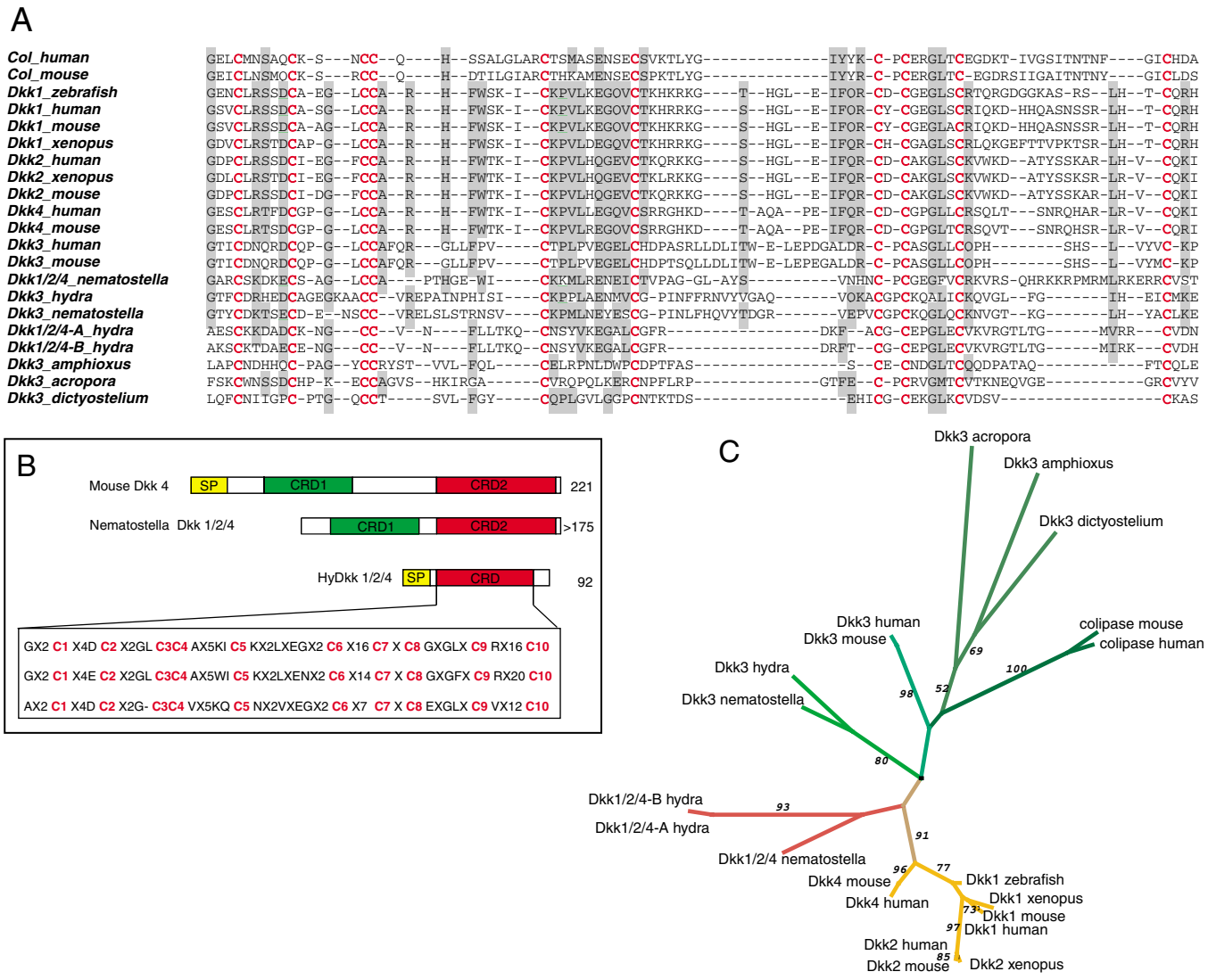

Fig. 2. Sequence analysis of *hydkk1/2/4*. (**A**) TCoffee alignment of HyDkk1/2/4 with the CRD2 of available Dkk molecules (see Materials and methods). (**B**) Domain structure of mouse Dkk4, NvDkk1/2/4 and HyDkk1/2/4; shaded boxes indicate conserved residues within CRD2; cysteines in red. (**C**) IQPNNI ML tree of Dkk CRD2 domains with TREE-PUZZLE support values (>50).

pattern in the temperature-sensitive *Hydra magnipapillata* strain sf-1. This mutant loses interstitial stem cells after heat shock at 28°C and, over time, all non-dividing derivatives, i.e. nematocytes and neurons, but also gland cells disappear (Sugiyama and Fujisawa, 1978). As expected, *hydkk1/2/4*-expressing gland cells were lost after a heat shock (Fig. 3C). Because gland cells have a limited proliferating capacity (Schmidt and David, 1986), they are lost more slowly than nematocytes and nerve cells. Thirty days after heat shock, the number of *hydkk1/2/4*-expressing gland cells diminished by more than 90% to about 50. These residual *hydkk1/2/4* cells were full of vesicles (Fig. 3D). We also found a downregulation of *hydkk1/2/4* expression in gland cells at sites of oogenesis (Fig. 3E-G), but not spermatogenesis (Fig. 3H). This is in accordance with their i-cell origin, as oogenesis is accompanied by the differentiation of i-cells into nurse cells.

hydkk1/2/4 expression during budding

As HyDkk1/2/4 is a putative antagonist to the Wnt signalling pathway in Hydra, we analyzed the expression pattern of *hydkk1/2/4* in budding polyps. Surprisingly, there were no changes in the expression level of *hydkk1/2/4* in the entire bud, neither in early, nor in mid-bud stages (Fig. 4A). Only in late stages, just before the tentacles emerge, was *hydkk1/2/4* downregulated at the site of head formation, reflecting the expression pattern in detached polyps. It remains unclear whether this decrease in *hydkk1/2/4* signal is due to transcriptional downregulation, the active retreat of gland cells or apoptosis.

hydkk1/2/4 expression during regeneration

During regeneration the transcriptional regulation of *hydkk1/2/4* is highly dynamic. In animals bisected at 80% body length, *hydkk1/2/4* expression was markedly upregulated at the site of cutting within 30 minutes (Fig. 4). High levels of expression were sustained up to 6 hours after head removal in 50% of all animals (Fig. 4B,C), which is significantly longer than the time required for wound closure (1 hour). Afterwards, *hydkk1/2/4* was completely downregulated in the presumptive head (Fig. 4B). With the emergence of tentacles, 30 hours after head removal, the apical region of all regenerates was free of *hydkk1/2/4* expression.

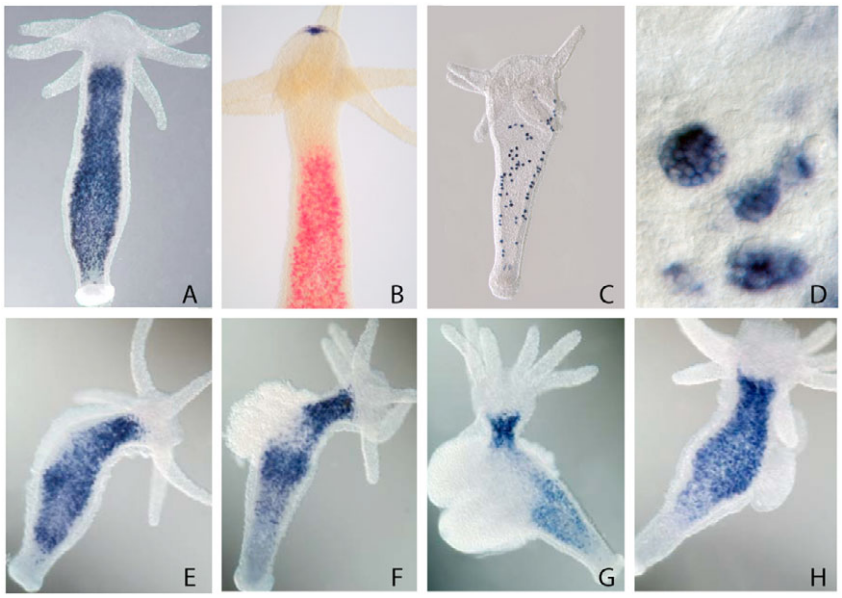

Fig. 3. *hydkk1/2/4*-expression analysis by whole-mount in situ hybridization. (**A**) Whole animal. (**B**) Double ISH: *hywnt3a* (blue) and *hydkk1/2/4* (red). (**C**,**D**) Residual gland cells in epithelial animals of strain sf-1 after heat shock; individual gland cells are shown in D. (**E-H**) Animals undergoing gametogenesis. (E-G) Oogenesis; (H) spermatogenesis.

The region of stimulated *hydkk1/2/4* expression measured roughly 5-10 epithelial cell diameters, which corresponds to 10% of the entire body length (Fig. 4B, 0.5 and 4 hours). ISH on macerated cells revealed that the number of *hydkk1/2/4*-expressing gland cells increased at the site of regeneration. We determined the ratio of *hydkk1/2/4*-expressing cells to epithelial cells in uncut control animals and in isolated regenerating tips (correspond to 10 % of the entire body length) 1 hour after head removal. This ratio doubled from 0.33±0.12 in uncut control animals to 0.62±0.06 at the site of regeneration. The expression level in small and large gland cells was also markedly elevated in the regenerating tip when compared with control tissue from the body column (Fig. 4D,E). *hydkk1/2/4* expression was also upregulated in foot regeneration, but the number of *hydkk1/2/4*-expressing cells was not as high as in head regenerates (data not shown). Thus, regeneration induces a dramatic increase in *hydkk1/2/4* expression at the site of tissue repair.

hydkk1/2/4 is involved in the injury response

The rapid increase of *hydkk1/2/4* message during head regeneration could be either caused by the removal of inhibiting signals emanating from the head or by the injury stimulus itself. We tested both possibilities. (1) Animals were injured by making a deep cut into the body column and leaving the head intact. Injured animals exhibited a strong stimulation of *hydkk1/2/4* transcripts for at least 6 hours at the site of injury (Fig. 5A). Even after wound closure, increased levels of *hydkk1/2/4* transcripts were still recognizable in cells at the site of the wound. (2) Heads of polyps were removed without wounding by tying a knot with a thin hair around the polyp's subhypostomal region (Newman, 1974). No significant upregulation of *hydkk1/2/4* expression occurred in such polyps (Fig. 5B). In agreement with Newman's observations, tied regenerates did not regenerate normally. In conclusion, our results suggest that it is the injury signal itself that causes a stimulation of *hydkk1/2/4* expression, and not the removal of an inhibitory signal from the head.

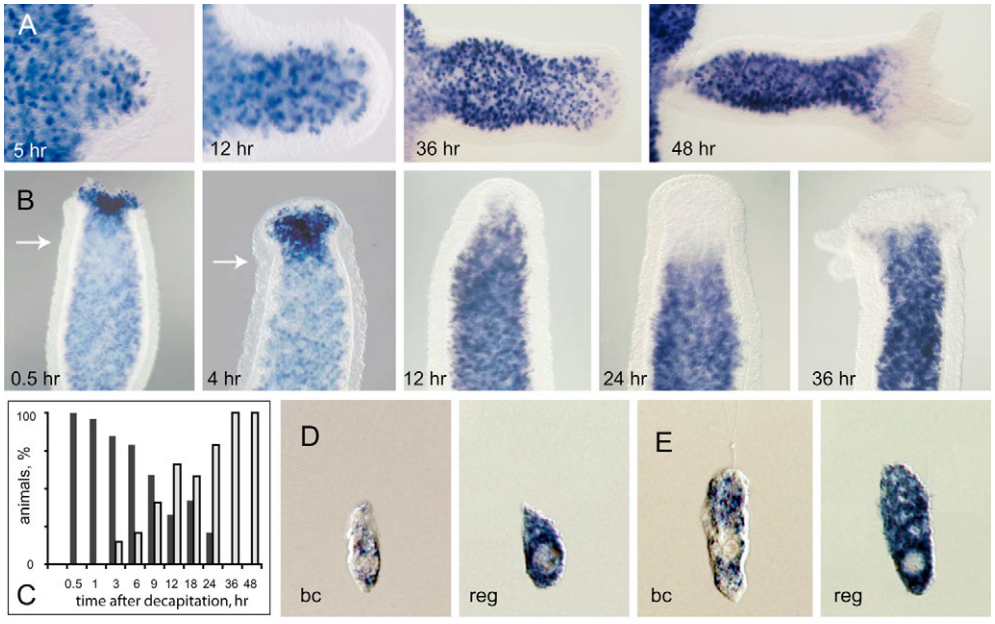

Fig. 4. *hydkk1/2/4* expression during budding and regeneration. (**A**) Early to late developing bud stages; (**B**) head regeneration. Polyps were decapitated at 80% body length and allowed to regenerate for the times indicated; arrows indicate the cutting position to isolate regenerating tips for quantification (see text). (**C**) Quantification of *hydkk1/2/4*-expression dynamics in regenerating tips. Expression in the apical 10% of body length was determined (n=14 to 24 polyps per sample): dark bars represent animals with *hydkk1/2/4* upregulation, light bars represent silenced *hydkk1/2/4* expression. (**D**,**E**) *hydkk1/2/4* expression in macerated cells. bc, cells of normal body column tissue; reg, cells from regenerating tissue.

hydkk1/2/4 is involved in the loss-of-regeneration capacity of epithelial *Hydra*

To assess further the relevance of HyDkk1/2/4 for regeneration, we analyzed the mutant strain sf-1, which loses *hydkk1/2/4*-expressing gland cells after long-term starvation (Fig. 6). We found that the regeneration capacity of heat-shocked, starved sf-1 animals was strongly correlated to the depletion of *hydkk1/2/4*-expressing gland cells. The number of *hydkk1/2/4*-expressing cells was reduced from an average of 270 cells at t_0 to four cells at t_{46} (Fig. 6A). The regenerative capacity of these animals was severely reduced: the number of regenerated tentacles dropped from 6.1 at t_0 to 0.4 at t_{46} (Fig. 6A). Non-heat-shocked control polyps lost only about 20% of their *hydkk1/2/4*-expressing cells and continued to regenerate fairly normally (at t_{35}, 3.9±1.3 tentacles in sf-1 and 3.9±1.1 in 105 animals).

Figure 6 shows representative examples of 35-day-starved animals at 4 hours after head removal (Fig. 6B-D) and at 9 days of regeneration (Fig. 6E-G). Most regenerating pieces had no *hydkk1/2/4*$^+$ *cells* (Fig. 6B), although a few had up to 50 *hydkk1/2/4*$^+$ cells (Fig. 6C,D). Following regeneration, almost all 'regenerates' lacked *hydkk1/2/4*$^+$ cells and failed to regenerate properly. Very few pieces regenerated a head (Fig. 6G). Although most of these regenerates lacked *hydkk1/2/4*$^+$ cells (Fig. 6G), we presume that such successful regenerates had *hydkk1/2/4*$^+$ cells at the start of regeneration.

We also tested *wnt* expression in *hydkk1/2/4*-depleted animals. We found a significant number of ectopic *hywnt3a* expression domains in such animals (Fig. 6H-J), but never in non-heat-shocked control animals that were starved for the same time (data not shown). In 47% of all analyzed *hydkk1/2/4*-depleted sf-1 polyps (n=55), several different-sized patches of *hywnt3a*-expressing cells were formed along the body column. This suggests that in a *hydkk1/2/4*-depleted background *hywnt3a* expression can be spontaneously activated. These are unexpected data, indicating that *hydkk1/2/4* gland cells are required for normal regeneration in *Hydra* and have a function in the regulation of *hywnt3a* gene expression (see Discussion).

Inhibition of *hydkk1/2/4 expression* in alsterpaullone-treated polyps

We tested the putative antagonism between Wnt and *hydkk1/2/4* gene expression in an additional experiment. We created a *hywnt3a* overexpression situation by treating polyps with the kinase inhibitor alsterpaullone (Knockaert et al., 2002; Leost et al., 2000). Alsterpaullone specifically inhibits Gsk3 in *Hydra* and thereby activates the canonical Wnt signalling pathway (Broun et al., 2005).

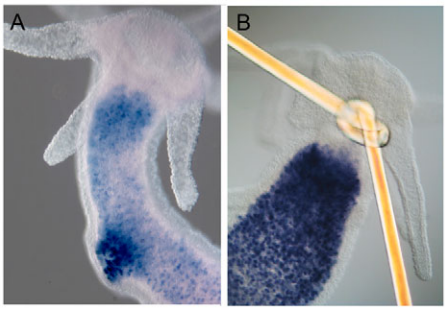

Fig. 5. *hydkk1/2/4*-expression dynamics. (**A**,**B**) *hydkk1/2/4*-expression dynamics in injured (A) and ligated (B) animals after 6 hours and 1 hour, respectively.

Alsterpaullone-treated polyps (24 hours) formed numerous spot-like *hywnt3a*-expression domains after 3 days (see Fig. S2 in the supplementary material), followed by ectopic tentacles after 3-4 days (Fig. 7A,B) and ectopic head-like structures after 8 days (Fig. 7A,B). *hydkk1/2/4* transcription levels successively decreased 24-48 hours after the onset of alsterpaullone treatment, and were completely absent when ectopic tentacles were formed (Fig. 7B,C). Double ISH indicates that the downregulation of *hydkk1/2/4* starts from the centre of the *hywnt3a*-expression domains in the body column (Fig. S2 in the supplementary material). At later stages, *hydkk1/2/4* expression was restored in the tentacle-free tissue between distinct heads (Fig. 7C). Thus, *hydkk1/2/4* expression is negatively regulated by canonical Wnt signalling.

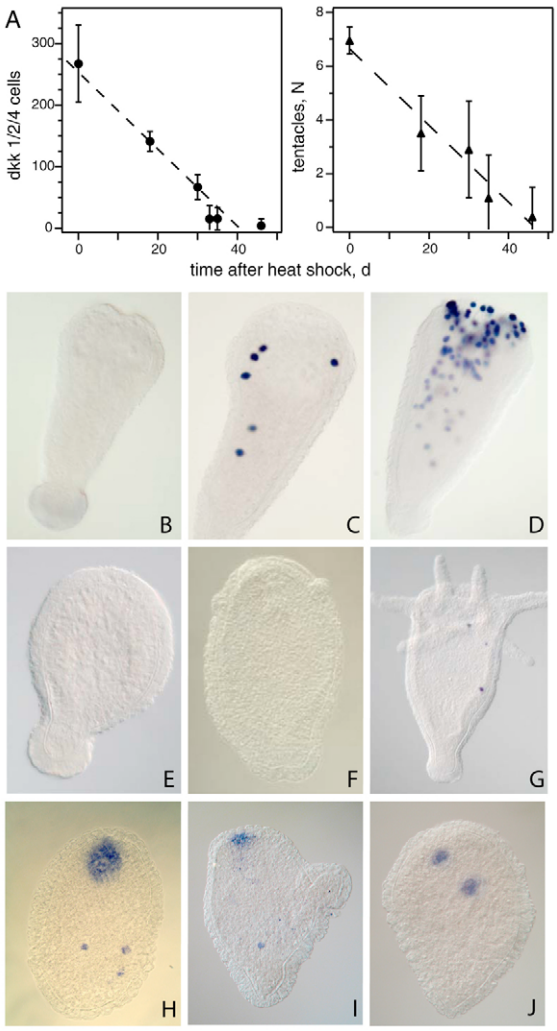

Fig. 6. *hydkk1/2/4 and hywnt3a* expression in regenerating epithelial sf-1 polyps. (**A**) Quantification of residual *hydkk1/2/4*-positive gland cells in body column pieces (left); n=6, 3, 5, 48, 37 and 22 with increasing time. Efficiency of head regeneration was measured as the average number of tentacles per regenerate (right); n=20, 101, 69, 178 and 140. Bars indicate s.d. Animals were cut at 50% body length and regenerated at least 4 hours prior to in situ hybridization and up to 9 days for determination of regeneration behaviour. (B-G) *hydkk1/2/4* expression in individual regenerates, 4 hours (**B-D**) and 9 days (**E-G**) after head removal. (**H-J**) Ectopic *hywnt3a* expression in regenerating epithelial sf-1 polyps (5-9 days regeneration).

HyDkk1/2/4 is functionally active in *Xenopus*

Overexpression is an instructive experiment to elucidate protein function. Because *hydkk1/2/4* overexpression in *Hydra* was not possible, we have chosen a heterologous approach in *Xenopus laevis*. As demonstrated by Glinka et al. (Glinka et al., 1998), endogenous *xdkk1* overexpression in the embryo leads to enlarged head structures and reduced posterior structures (Dickkopf phenotype) due to enhanced inhibition of late canonical Wnt signalling. Injection of *hydkk1/2/4* mRNA in each blastomere of four-cell embryos resulted in severe morphological defects comparable to the Dickkopf phenotype (Fig. 8A): anterior structures, like the cement gland, were enlarged, whereas posterior trunk regions were severely reduced if not abolished. To further demonstrate the Wnt-inhibitory activity of *hydkk1/2/4*, we co-injected *hydkk1/2/4* with *xwnt8*, which induces secondary axis formation in *Xenopus* embryos. This secondary axis formation could be blocked by the co-injection of *hydkk1/2/4*, even though it was less effective than *xdkk1* (Fig. 8B). These experiments clearly show that HyDkk1/2/4 is a functional homologue of vertebrate Dkk1 and Dkk4. To determine the inhibiting effect of HyDkk1/2/4 on downstream targets of canonical Wnt signalling, an animal cap assay was performed using *siamois*, a direct Wnt target gene, as readout (Fig. 8C). Embryos were injected with *xwnt8* mRNA in presence or absence of *xdkk1* and *hydkk1/2/4* mRNA. RT-PCR was then carried out on animal caps to measure the expression of *siamois*. Detection of *brachyury* and *histone-4* served as controls for mesodermal contamination and internal normalization, respectively. Non-injected control embryos showed no detectable *siamois* transcripts. *siamois* was upregulated upon *xwnt8* injection, and this upregulation was abolished by co-injection with *xdkk1* or *hydkk1/2/4*.

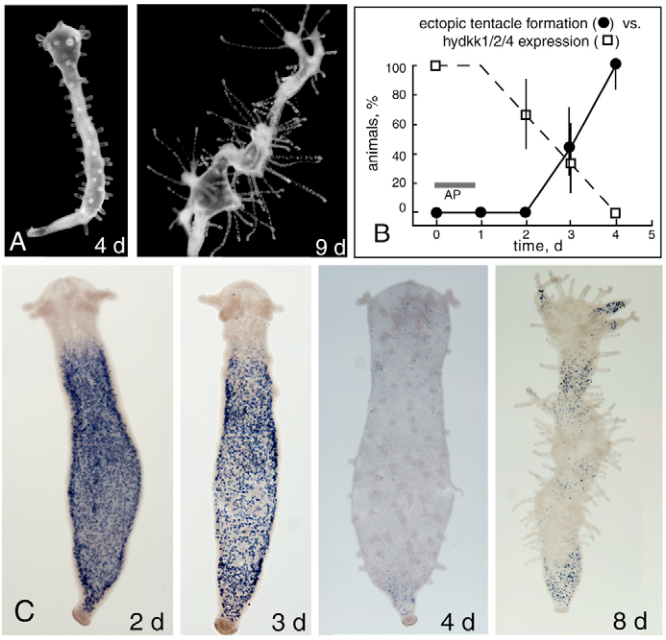

Fig. 7. *hydkk1/2/4* expression in alsterpaullone (AP)-treated animals. Polyps were incubated for 24 hours in 5 μM AP and then transferred to *Hydra* medium for time indicated. (**A**) Dark-field micrographs. (**B**) Quantification of *hydkk1/2/4* expression (white squares) and tentacle formation (black circles) from three independent experiments, determined as the percentage of *hydkk1/2/4*-expressing polyps and the number of tentacles from a total of 120 polyps. Bars indicate s.d.; solid line indicates the length of AP treatment. (**C**) ISH with an antisense *hydkk1/2/4* probe (for details see Results).

This result was further verified using the TOPFLASH Wnt reporter (Korinek et al., 1997) (Fig. 8D). In this assay, HyDkk1/2/4 showed a dose-dependent, Wnt-inhibitory activity. These findings strongly indicate that HyDkk1/2/4 is a Dickkopf homologue capable of inhibiting Wnt signalling.

DISCUSSION

The origin of the Dkk proteins in metazoan evolution

Dickkopf proteins have been identified as major inhibitors of Wnt signalling in vertebrates. Among the four subfamilies only Dkk1, Dkk2 and Dkk4 are inhibitory ligands of the Lrp5/Lrp6-Wnt-Frizzled complex; Dkk3 does not inhibit Wnt signalling (Wu et al., 2000; Brott and Sokol, 2002). Here, we describe, for the first time in an invertebrate, the identification of a novel Dickkopf-related protein, which has an inhibitory function in Wnt signalling.

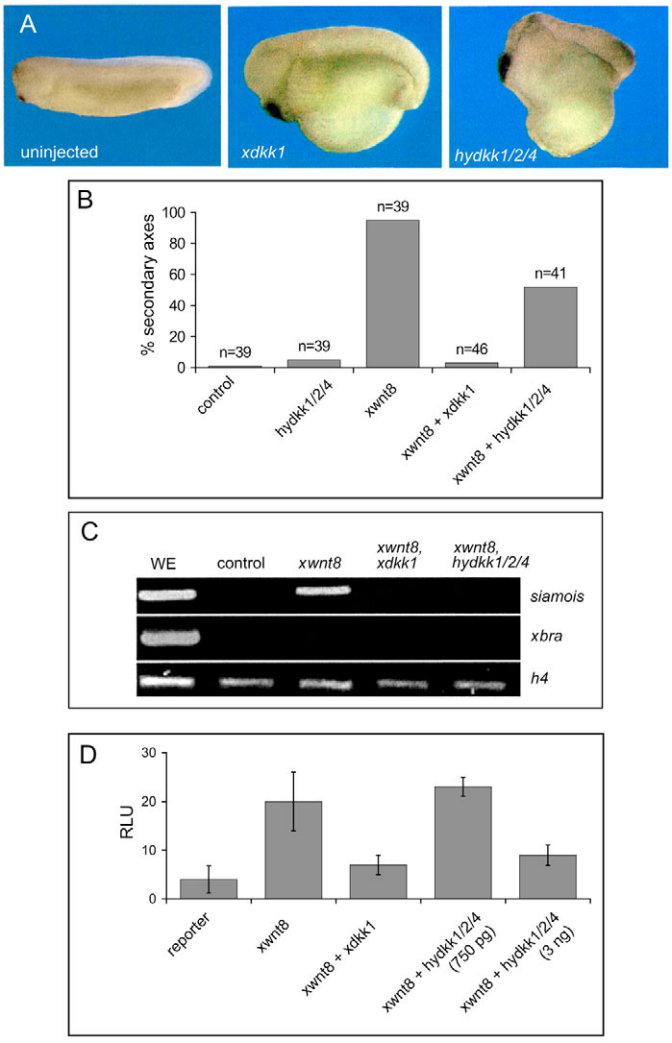

Fig. 8. Heterologous expression of *hydkk1/2/4* in *Xenopus laevis* embryos. (**A**) Overexpression of *xdkk1* or *hydkk1/2/4* induces the Dickkopf phenotype. (**B**) Inhibition of *xwnt8*-mediated secondary axis induction by *xdkk1* or by *hydkk1/2/4* co-injection. (**C**) Animal cap assay. Inhibition of *siamois* induction by co-injection of *xdkk1* or *hydkk1/2/4* was assayed by RT-PCR. WE, whole embryos; xbra, *Xenopus brachyury*; h4, *histone-4*. (**D**) Co-injection of *hydkk1/2/4* (750 pg and 3 ng) or *xdkk1* blocks *xwnt8*-induced activation of a *siamois-luciferase* reporter construct (stage 10 to 10.5).

Structurally, the novel protein corresponds to the CRD2 of vertebrate Dkk1, Dkk2 and Dkk4; the similarity is highest to mammalian Dkk1 and Dkk4. There is a second *hydkk1/2/4* gene in the *Hydra* EST database (CN559480; *Hydra* EST-Kiel) with more than 79% nucleotide identity (probably a product of a recent hydrozoan-specific gene duplication). In *Nematostella vectensis* there is a *dkk1/2/4* gene that contains the N-terminal CRD1. We presume that the CRD1 of this gene is lost in the *Hydra hydkk1/2/4*. There is also a *dkk3* gene in *Nematostella*, which is related to the nematocyte-specific *hydkk3* in *Hydra* (Fedders et al., 2004).

Based on their conserved CRD2, all cnidarian *dkk* genes share features of chordate *dkk3* and *dkk1/2/4* genes. The significant diversification of cnidarian *dkk* genes suggests a deep split into *dkk3* and *dkk/1/2/4* gene families during early metazoan evolution. Although this could not be clarified with certainty by phylogenetic analysis because of the high divergence of the sequences, this notion is supported by the chromosomal location of vertebrate Dkks. An analysis of paralogy regions in the human genome using the paralogon database Human 5.28 of Wolfe and McLysaght (Luke et al., 2003; Lundin, 1993; McLysaght et al., 2002) (http://wolfe.gen.tcd.ie/dup/human5.28/) shows that human *DKK1*, *DKK2* and *DKK4* are located within related chromosomal regions (*DKK1* maps to 10q11, *DKK2* to 4q25 and *DKK4* to 8p11). These sites lie within the well-characterized 4/5/8/10 paralogy group, which also contains FGF receptors and NK homeobox genes (Birnbaum et al., 2000; Coulier et al., 2000a; Coulier et al., 2000b; Leveugle et al., 2004; Pollard and Holland, 2000), and numerous other duplicated gene families. Genes within this paralogy region were duplicated early in vertebrate evolution (Pollard and Holland, 2000; Luke et al., 2003). Hence, we conclude that *dkk1*, *dkk2* and *dkk4* most likely originated by gene duplication. By contrast, human *DKK3* maps to 11p15.3, which is not part of the same set of paralogy regions. This finding is consistent with the hypothesis that the features common to the *Hydra*, *Nematostella* and vertebrate *dkk1/2/4* subgroup were likely to have been present in the common ancestor of cnidarians and bilaterians.

hydkk1/2/4 is an early regeneration-responsive gene

The most obvious role HyDkk1/2/4 plays in *Hydra* is its function during early regeneration. We found a rapid and dramatic increase of *hydkk1/2/4* message at the site of injury within the first hour after head removal. *hydkk1/2/4* upregulation was clearly related to the injury stimulus, as it also occurred by simply cutting the animals at any site in the body column. This early upregulation seems to be indispensable for regeneration, because animals whose heads were removed by means of the ligature technique (Newman, 1974), with minimal or even without injury, exhibit no *hydkk1/2/4* upregulation and could not regenerate normally. Furthermore, animals that have lost *hydkk1/2/4*-expressing cells also lose their regeneration capacity.

The regeneration deficient mutant strain reg-16 is also sensitive to the injury stimulus: regenerates develop normally after setting a second cut at the site of injury (Sugiyama and Fujisawa, 1977). We therefore presume that an essential trigger for head regeneration in *Hydra* is the early release of Dickkopf proteins at the site of cutting. This role of gland cells in patterning processes has certainly been underestimated so far.

Interestingly, Prockop et al. (Prockop et al., 2003) found in the process of mammalian tissue repair that marrow stromal cells (MSCs) from the bone marrow secrete and require Dkk1 for cell expansion in vitro and during the process of tissue repair. It probably interacts with *Wnt5a* in the growth regulation of MSCs (Gregory et al., 2005; Gregory et al., 2003; Horwitz, 2004; Prockop et al., 2003). *Dkk1* has also been found to be strongly upregulated at the sites of apoptosis during vertebrate limb development, and in UV-irradiated tissue (Grotewold and Ruther, 2002a; Grotewold and Ruther, 2002b). Because regeneration in *Hydra* is accompanied by dramatic changes in the pattern of cell cycle and proliferation at the site of cutting (Holstein et al., 1991; Holstein et al., 2003), we propose that HyDkk1/2/4 in *Hydra* has a similar function in the response to stress signals and the initiation of tissue repair. It is as yet unclear which signal actually causes the extremely fast upregulation of *hydkk1/2/4* transcripts. One molecular trigger could be Jun (also known as c-Jun), a stress-responsive transcription factor and activator of *dkk1* that is upregulated during embryonic wounding (Grotewold and Ruther, 2002a; Grotewold and Ruther, 2002b). Another candidate is β-Catenin, which can also activate *dkk1* expression in a dose-dependent manner (Gonzalez-Sancho et al., 2005; Niida et al., 2004). Both genes have been identified in *Hydra* (Hobmayer et al., 2000).

Our data show that there exists an additional level of complexity in the regulation of *hydkk1/2/4* expression. We found during late regeneration, i.e. in late bud stages and after activation of the Wnt/β-catenin pathway by treatment with alsterpaullone, a complete silencing of *hydkk1/2/4* expression. This clearly indicates that the early activation of *hydkk1/2/4* expression during regeneration is only transient. The shift in the transcriptional regulation of *hydkk1/2/4* found during *Hydra* regeneration shares similarities with the transcriptional downregulation of *dkk1* in human colon tumours. Colon tumours exhibit activated Wnt/β-Catenin signalling and downregulation of *dkk1* expression (Gonzalez-Sancho et al., 2005). It was proposed that hypermethylation of the *dkk1* promotor leads to *dkk1* silencing, similar to the silencing of other Wnt inhibitory genes and to *dkk3* (Suzuki et al., 2004; Caldwell et al., 2004; Gonzalez-Sancho et al., 2005). Thus, in *Hydra*, *hydkk1/2/4* expression appears to be stably silenced by Wnt/β-Catenin signalling in the head, while it might suppress Wnt/β-Catenin signalling in the body column, generating the distinct compartments of *Hydra*'s body, as implied by the sharp boundary underneath the head.

HyDkk1/2/4 is an evolutionary conserved antagonist of Wnt signalling

We tested the function of HyDkk1/2/4 as a putative Wnt inhibitor in *Xenopus* embryos. In this heterologous system, HyDkk1/2/4 has similar Wnt-inhibitory properties to endogenous XDkk1. (1) HyDkk1/2/4 and the endogenous XDkk1 have the same anteriorizing capacity in *Xenopus* embryogenesis. (2) HyDkk1/2/4 can block XWnt8-induced secondary axis formation (Fig. 8B). (3) HyDkk1/2/4 blocks the induction of the downstream target gene of the canonical Wnt pathway *siamois* in animal cap assays. The fact that HyDkk1/2/4, corresponding to the carboxy-terminal cysteine-rich domain of Dkks, can exert an inhibitory effect on Wnt signalling is consistent with similar findings for the CRD2 of vertebrates. It was also shown that the CRD2 of Dkk4 is proteolytically cleaved from the full-length protein (Krupnik et al., 1999), which might represent an ancient feature of the basal Dkk4 within vertebrates (Fig. 2C). By comparison, Dkk3 has a linker sequence between CRD1 and CRD2 without a proteolytic cleavage site, and has not been shown to inhibit or modulate Wnt signalling at all (Krupnik et al., 1999; Mao and Niehrs, 2003).

The evolutionary origin of Dickkopf proteins remains unclear, but cnidarians suggest a possible scenario. The CRD2 is similar to colipases, which facilitate the interaction of pancreatic lipases with

lipid micelles (Krupnik et al., 1999). Based on this structural similarity, it was proposed that the CRD2 of Dkk could help other proteins to interact with lipids in order to regulate Wnt function (Aravind and Koonin, 1998). Wnt proteins are indeed tightly associated with the cell surface (Nusse, 2003; Smolich et al., 1993). Wnts are palmitoylated proteins and are therefore much more hydrophobic than is predicted from their primary amino acid sequences (Nusse, 2003; Willert et al., 2003). Thus, lipid binding of Dkk may have initially served to tether Wnt ligands to target membranes. From such a facilitated interaction of Wnt and Dkk at the plasma membrane, the Dkk/Wnt antagonism may have evolved. In accordance with this idea, we found that HyDkk1/2/4 enhances human LRP6- and mouse Wnt1-induced Wnt signalling in 293T cells 1.5- to 2-fold (see Fig. S3 in the supplementary material), similar to Dkk2 in *Xenopus* embryos (Brott and Sokol, 2002).

The evidence for an inhibitory function in *Hydra* is less clear. Nevertheless, the expression patterns of *hydkk1/2/4* and *hywnt3a/hyβ-catenin/hytcf* are mutually exclusive and suggestive: *hywnt3a* and other Wnt genes from *Hydra* are expressed in the hypostomal region around the mouth of the *Hydra* (F. Rentzsch, C.G., B.H. and T.W.H., unpublished) (Hobmayer et al., 2000), whereas *hydkk1/2/4* is uniformly expressed in the entire body column, but not in the hypostomal region (Fig. 3). This is consistent with the idea that HyDkk1/2/4 is involved in a mechanism that might help to suppress the expression of Wnt genes in the body column. It is also consistent with the fact that, in polyps that were depleted of *hydkk1/2/4*-expressing cells, *hywnt3a* is expressed in small cell clusters all over the body column. This patchy upregulation of *hywnt3a* was never observed in normal polyps except in the budding region. It should be emphasized, however, that this interaction might be more complicated. During budding, *hywnt3a*-expression spots regularly arise in a tissue that strongly expresses *hydkk1/2/4* (Hobmayer et al., 2000). *hywnt3a* and *hydkk1/2/4* are also co-expressed during early regeneration. Thus, although our functional assays in *Xenopus* provide clear evidence that the HyDkk1/2/4 molecule has the ability to antagonize Wnt signalling, it remains unclear as to what extent HyDkk1/2/4 actually antagonizes Wnt signalling in *Hydra*.

Evolutionary considerations

Although *hydkk1/2/4* is evidently required for the regeneration process in *Hydra*, it remains to be clarified whether the molecule has an additional role in steady-state animals. At this point, we can only speculate, as no experiments addressing this question have been done. However, the characteristic expression pattern of *hydkk1/2/4* and the fact that *hydkk1/2/4* can induce the Dkk1 phenotype in *Xenopus* suggests that *hydkk1/2/4* has a similar function in steady-state *Hydra* polyps as during head and neuronal induction in vertebrates. In vertebrates, Dkk1 can induce secondary heads (Glinka et al., 1997), and is necessary to activate neural genes in the anterior and dorsal region of the vertebrate brain (Glinka et al., 1998; Kazanskaya et al., 2000; Mukhopadhyay et al., 2001; Niehrs, 2004). In *Hydra*, *hydkk1/2/4* is only expressed in the body column. Figure 9 shows schematically that in this region the neuronal differentiation from multipotent interstitial stem cells takes place (Grens et al., 1996; Lindgens et al., 2004; Smith et al., 1999; Technau et al., 1996). We therefore favour the definition of the body column as being the neurogenic region of the *Hydra*. We presume that the release of HyDkk1/2/4 from gland cells in the endoderm promotes neuronal differentiation by inhibiting the β-Catenin/Wnt signalling pathway in the ectoderm of the *Hydra* body column (Fig. 9A). The BMP antagonist Chordin is also expressed in the same tissue (F. Rentzsch, C.G., B.H. and T.W.H., unpublished), supporting the hypothesis that a primary function of Bmp and Wnt antagonism in metazoan evolution is the facilitation of neuronal differentiation. This hypothesis is supported by the expression patterns of cnidarian Wnt

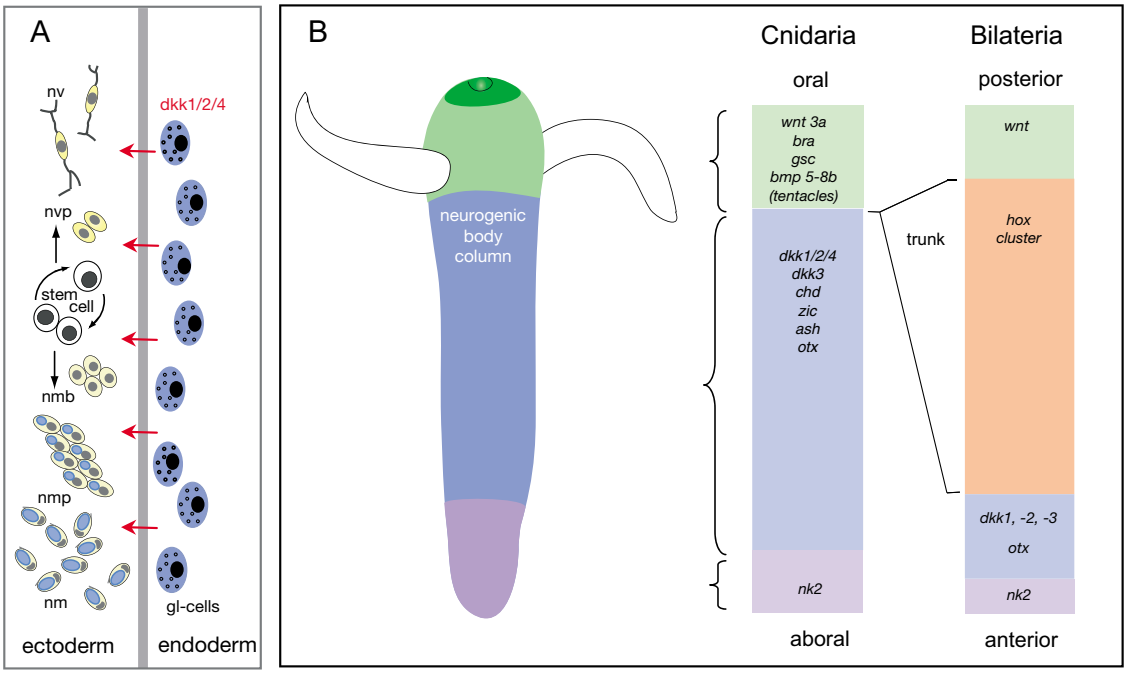

Fig. 9. *hydkk1/2/4*-expression pattern and neuronal differentiation in *Hydra*. (**A**) The release of HyDkk1/2/4 from gland cells in the endoderm is postulated to facilitate stem cell growth and the formation of neuronal precursor cells in the ectoderm. nvp, nerve cell precursors; nm, mature nematocytes; nmb, nematoblast; nmp, nematocyte precursors; nv, neuronal cells. (**B**) The *hydkk1/2/4*-expression domain correlates to that of neuronal and pro-neuronal genes in *Hydra*. Modified, with permission, from Meinhardt (Meinhardt, 2002; Meinhardt, 2004).

genes (Hobmayer et al., 2000; Kusserow et al., 2005) and by theoretical considerations on midline formation during the evolution of bilaterians (Meinhardt, 2002; Meinhardt, 2004). According to that idea, the *Hydra* body column is the counterpart to the vertebrate brain, where proneuronal and neuronal genes are expressed, whereas the bilaterian trunk evolved later, and intercalated between the posterior and anterior end.

Conclusion

The vital role of the expression of *hydkk1/2/4* in gland cells for head regeneration in *Hydra* was unexpected. Furthermore, despite the lack of phylogenetic information because of the high divergence of Dkks, the chromosomal location of vertebrate Dkks, the inversely related expression patterns of *hydkk1/2/4* and *hywnt3a* in *Hydra*, and the functional antagonism of HyDkk1/2/4 and canonical Wnts in *Xenopus* and *Hydra* are all consistent with the hypothesis that the Dkk/Wnt antagonism was already present in the last common ancestor of cnidarians and bilaterians. It thus appears that *Caenorhabditis* and insects have lost the Dkk-Wnt-antagonism, together with other developmental genes.

We thank Charles N. David (Munich) for critically reading the final version of this manuscript. We also thank Rebecca Furlong and Peter Holland (Oxford) for their essential help with the phylogenetic analysis and for providing the chromosome analysis for mammalian Dkks; Uli Technau (Darmstadt, Bergen) for sharing unpublished data and helpful discussions; Anne Lehmkuhl (Darmstadt) and Kirsten Wehner (Darmstadt) for technical assistance; and Hans Meinhardt for critically reading an early version of the manuscript. This work was funded by the DFG.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/4/901/DC1

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