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## A *Dickkopf-3*-related gene is expressed in differentiating nematocytes in the basal metazoan *Hydra*

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**Abstract** In vertebrate development the Dickkopf protein family carries out multiple functions and is represented by at least four different genes with distinct biological activities. In invertebrates such as *Drosophila* and *Caenorhabditis*, *Dickkopf* genes have so far not been identified. Here we describe the identification and characterization of a *Dickkopf* gene with a deduced amino acid sequence closely related to that of chicken *Dkk-3* in the basal metazoan *Hydra*. *HyDkk-3* appears to be the only *Dickkopf* gene in *Hydra*. The gene is expressed in the gastric region in nematocytes at a late differentiation stage. In silico searches of EST and genome databases indicated the absence of *Dkk* genes from the protostomes *Drosophila* and *Caenorhabditis*, whereas within the deuterostomes, a *Dkk-3* gene could be identified in the genome of the urochordate *Ciona intestinalis*. The results indicate that at an early stage of evolution of multicellularity Dickkopf proteins have already played important roles as developmental signals. They also suggest that vertebrate *Dkk-1*, *2* and *4* may have originated from a common ancestor gene of *Dkk-3*.

**Keywords** *HyDkk3* · *Dickkopf* · *Hydra* · Nematocytes · Cell differentiation

### Introduction

Proteins of the Dickkopf family have been implicated as critical molecular signals in development. *Dickkopf* genes belong to a multigene family that is found throughout

vertebrate species with at least four members in humans (Glinka et al. 1998; Monaghan et al. 1999; Krupnik et al. 1999). A characteristic feature of all members of the gene family are two cysteine-rich domains, separated by a certain number of amino acids (Glinka et al. 1998; Krupnik et al. 1999). Each of these domains contains ten conserved cysteine residues. In *Xenopus* embryos *Dkk-1* is expressed in the Spemann organizer region and is essential for head development (Glinka et al. 1998) by acting as antagonist of Wnt signaling. In mouse, *Dkk-1* is an essential inducer for head induction and is involved in distal limb patterning (Mukhopadhyay et al. 2001). *Dkk-2* can act as either agonist or antagonist of the Wnt signaling pathway (Mao and Niehrs 2003) and *Dkk-4* appears to have a similar function to *Dkk-1* in Wnt inhibition. Little is known about *Dkk-3* which has been suggested as “having a function very divergent from that of other *Dkks*” (Mao and Niehrs 2003). While Dickkopf proteins appear to play a conserved role as developmental signals within the vertebrates, conservation of this signaling process has not yet been demonstrated. No invertebrate *Dkk* gene has yet been reported (Hino et al. 2003). Indeed, it has been argued that *Dkk-1*, an instrumental factor in anterior fate determination in *Xenopus* and mouse, may represent a vertebrate-specific innovation.

A member of the phylogenetically old metazoan phylum Cnidaria is the freshwater polyp *Hydra* which has a diploblastic body plan consisting of only two epithelia, the ectoderm and the endoderm, surrounding a gastric cavity. There are about 20 cell types distributed among 3 cell lineages (for review see Bosch 2003). Each of the epithelial layers is made up of a cell lineage, while the remaining cells are part of the interstitial cell lineage which reside among the epithelial cells of both layers. Multipotent interstitial stem cells give rise to neurons, secretory cells and gametes in a position-dependent manner (Bosch and David 1987). These stem cells also give rise to nematocytes, which are unique to and characteristic of all cnidarians (David and Gierer 1974; Holstein 1981; Tardent 1995). In the past, several elegant

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studies have addressed the cellular and biochemical basis of nematocyte differentiation and revealed that their differentiation is a highly complex, multistep process (reviewed in Tardent 1995). Nematocytes differentiate in clusters of 8–32 cells in the body region (David and Challoner 1974). Cells within clusters remain interconnected by cytoplasmic bridges. During differentiation each nematoblast produces a nematocyst capsule inside a secretory vesicle. Following capsule differentiation, the clusters of differentiating nematocytes break up into single cells that migrate towards the tentacles and become mounted in specialized tentacle epithelial cells, termed battery cells (David and Gierer 1974). Although extensive studies at the biochemical (Kurz et al. 1991; Koch et al. 1998; Engel et al. 2001, 2002; Szczepanek et al. 2002) and ultrastructural (Mariscal 1974; Holstein 1981; Holstein et al. 1994) level have revealed the morphogenesis of capsules, little is known about the factors that regulate nematocyte behavior once capsule differentiation is completed. Cnidocytes migrate individually towards the base of the tentacles where they get incorporated into one of the ectodermal epithelial cells (Tardent 1995). The signals guiding the nematocytes from the gastric region towards the tentacles are not known. It seems likely, however, that cell-cell communication by secreted factors is involved.

An essential part of the positional information system along the *Hydra* apical-basal body axis are peptides (Bosch and Fujisawa 2001). Recently, four peptides have been shown to be capable of inducing head- or foot-specific differentiation: HEADY, pedibin/Hym-346, and Hym-323 (Bosch and Fujisawa 2001). Although the 12-amino-acid peptide HEADY is a potent inducer of apical fate and also sufficient for head induction (Lohmann and Bosch 2000), its role as developmental signal in *Hydra* is not yet completely understood. Therefore, we are screening for target genes sensitive to the absence or presence of HEADY. In the present study, we report that during this attempt we unintentionally identified a *Hydra* gene showing similarity to chicken *Dickkopf3* (*Dkk-3*). Since the gene is expressed late during nematocyte differentiation, at a stage which is characterized by changes in morphology and behavior allowing extended cell migration towards the tentacles, this is the first demonstration that a member of the *Dkk* gene family is involved in controlling developmental processes outside the vertebrates.

## Materials and methods

### Animals

*Hydra magnipapillata* polyps were cultured according to standard procedures at 18°C.

### Differential display PCR and isolation of *HyDkk-3*

*HyDkk-3* was isolated from HEADY-treated polyps and poly(A)+ RNA which was subjected to the previously described (Liang and Pardee 1992; Lohmann et al. 1995) non-radioactive differential display PCR using primers T(12)GA and OPA-9 (Operon). A full-length cDNA sequence was obtained by 5' and 3' RACE PCR. For 5' RACE PCR, we used a Splinkerette approach and double-stranded cDNA (ds cDNA) generated by the SUPERScript plasmid system (Stratagene). A Splinkerette adaptor (top strand: 5'-CGAATCGTAA CCGTTCGTAC GAGAATTCGT ACGA-GAATCG CTGTCCTCTC CAACGAGCCA AGG-3'; bottom strand: 3'-AAAAACGTTT TTTTGTGTC GGTCC-5') was blunt-end ligated to the ds cDNA. This ligation mix was used as a template for the first "touch down" PCR using primers 5'-GAATCGTAA CCGTTCGTACG-3' and 5'-GAACATATTG GTTGAATAAA CTGG-3' and the PCR profile as follows: 94°C for 5 min, 3 times 7 cycles of 94°C for 30 s, 62°C down to 56°C for 40 s, 72°C for 2.5 min, plus 20 cycles of 94°C for 40 s, 56°C for 40 s, and 72°C for 2.5 min. For the second nested PCR, the product of the first PCR was diluted 1:50 and mixed with primers 5'-TACGAGAATC GCTGTCCTC-3' and 5'-CAACTTGCTT GCA-TATAAGC G-3' and submitted to a PCR with a profile of 94°C for 30 s, 56°C for 40 s, and 72°C for 2 min (Devon et al. 1995). For 3' RACE single strand cDNA was synthesized using the first strand cDNA synthesis kit (Amersham) and the anchor primer (5'-T<sub>17</sub>GACTCGAGTC GACATCGA-3'). For the 3' RACE PCR which followed, the adaptor primer (5'-GACTCGAGTC GACATCGA-3') and the *HyDkk-3* specific primer (5'-CAGAATGCC AATGCTGTGA A-3') were used.

### Molecular techniques

Nucleic acid isolation, cDNA cloning, and DNA sequence analysis were carried out following standard procedures. For Southern blot analysis, 10–15 µg genomic DNA was digested with different restriction endonucleases, separated on a 0.7% agarose gel and transferred onto Hybond N+, nylon membrane (Amersham). The hybridization probe of 563 bp corresponds to nucleotides 283 to 845 of the full length cDNA (Fig. 1). Sequencing the corresponding fragment from genomic DNA revealed the presence of an intron with a restriction site for *BclI*. Whole-mount in situ hybridization was carried out as described in Martinez et al. (1997) on *H. magnipapillata*, *H. oligactis*, and *H. vulgaris* (AEP) using 548-bp digoxigenin-labeled RNA probes corresponding to nucleotides 298 to 845 of the full length cDNA (Fig. 1).

### Bioinformatics

The predicted amino acid sequence of *HyDkk-3* was analyzed for signal peptide sequences with the help of the SignalP V2.0 program available at the homepage of the Center for Biological Sequence Analysis of the University of Denmark (<http://www.cbs.dtu.dk/services/SignalP-2.0/>). For alignment of multiple sequences of the *Dickkopf* family, sequences corresponding to cysteine rich domain 2 were analyzed using the Clustal W program (<http://www.ebi.ac.uk/clustalw/>). Evolutionary relationships were analyzed using the Neighbor-joining method (Saitou and Nei 1987) and viewed as a phylogenetic tree. The following sequences were taken from the National Center for Biotechnology Information (NCBI) server: *Dkk-1 Homo* (O94907), *Dkk-2 Homo* (Q9UBU2), *Dkk-3 Homo* (Q9UBP4), *Dkk-4 Homo* (Q9UBT3), *Dkk-3 Gallus* (Q90839), *Dkk-1 Xenopus* (AAC02427), *Dkk-2 Mus* (Q9QYZ8), *Dkk-4 Mus* (NP\_663567), *Colipase Oryctolagus* (AAA02911), *Dkk like cys2-1 Hydra* (consensus sequence of tac30c03.y1 and tab34a10.x1 at EBI), *Dkk-like cys2-2 Hydra* (consensus sequence of taa23c11.x2, taa23c11.y1 at EBI and BP510080 at DDBJ). One expressed sequence tag (EST) encoding *Ciona Dkk3* (cibd068a20) was identified in the *Ciona* EST database "Ghost Database" at <http://ghost.zool.kyoto-u.ac.jp/indexr1.html> by performing TBLASTN

## A

1 AGATTTTCATTAATAATTTTATGCTAGTCAAATATTTAT**TAAAAAATATA**TAA****AGATATATAACGAAA**ATGT**CGAAACTATTTATTATCTATACG  
**M S K L F I I Y T**

91 TTTTGTATTTTGTGGCGTATGCAGAAGACAAAAAGGTTCCAAAACCAGTCGCACCTTCGTTAGAAAAAAGAGGCGATATACAGCCTCGC  
F C I F V A Y A E D K K V P K P V A P S L E K R G D I Q P R

181 ATGCTTGGCTCCAGGATATTACTCTCAAGAATGTAACGCCACAAAGCTTGGCCCTGAGAAAAAATATTGCCATTTGTTTCTATGTGTTTCAT  
 M L A P G Y Y S Q E **C N A H K A C P E K K Y C H L F L C V H** **Cys-1**

271 TGCTTAAAAGAGAACGTAGCCTGCACACAGAATGGCCAATGCTGTGAAGGACAGTGTACTTATGGAAGATGTAAGCTGGTGTCTTGAA  
**C L K E N V A C T Q N G Q C C E G Q C T Y G R C** K A G V S E **Cys-1**

361 GGACAACCAGGAACATTTTGTGATAGACACGAGGATTGTGCTGGAGAAGGAAAAGCGGCTTGTGTGTAAGAGAACCAGCCATAAACCT  
 G Q P G T F **C D R H E D C A G E G K A A C C V R E P A I N P** **Cys-2**

451 CATATATCTATATGCAAGCCACCATTGGCTGAAAACATGGTTTGGCGACCAATAAACTTTTTT**CAGAAATGTTTACGTTGGAGCTCAAGTA**  
**H I S I C K P P L A E N M V C G P I N F F R N V Y V G A Q V** **Cys-2**

541 CAAAAAGCATGCGGACCTTGCAAAACAAGCGCTTATATGCAAGCAAGTTGGTCTTTTTTGGAAATTCACGAAATATGTATGAAGGAAGATGAT  
**Q K A C G P C K Q A L I C K Q V G L F G I H E I C M K E D D** **Cys-2**

631 AAAAAAGAA**TAG**ACACAAAGTAAATCCAAACATGTATCACTAGGTACT**TG**ATTACAAATTCACACCAAGCAAGCAAACACAAACA**ATAA**  
 K K K \*

721 TGTAA**TAA**ATGTACT**TGA**CAAATAATAGGAAGCAA**TAA**AATGTTTATACCCAGTTTATTCAACCAATATGTTCTAT**TAA**CGAAATTGG

811 TTTTCTCA**TAGA**ATAAGTATTTTGGCGTTATGTTT**TAA**AATAAATTTATATATAAAAAAAAAAAAAAAAA

## B

## Cys-rich domain 1:

|                  |     |   |     |
|------------------|-----|---|-----|
| <b>HDkk-3</b>    | 144 | SHE <b>CI</b> IDED <b>CG</b> PSMY <b>CQ</b> FAS <b>FQ</b> Y <b>TC</b> Q <b>PCR</b> G <b>Q</b> R <b>ML</b> C <b>TRD</b> SE <b>CC</b> GD <b>Q</b> L <b>CV</b> WG <b>HC</b> TKMATRGSNGTI         | 207 |
| <b>MDkk-3</b>    | 144 | SHE <b>CI</b> IDED <b>CG</b> PTRY <b>CQ</b> FSS <b>FK</b> Y <b>TC</b> Q <b>PCR</b> D <b>Q</b> ML <b>C</b> TRDSE <b>CC</b> GD <b>Q</b> L <b>CA</b> WG <b>HC</b> CTQKATKGGNGTI                  | 207 |
| <b>GDkk-3</b>    | 136 | NHE <b>CI</b> IDED <b>CET</b> G <b>KY</b> C <b>Q</b> FST <b>FE</b> Y <b>K</b> C <b>Q</b> P <b>CK</b> T <b>Q</b> HT <b>CS</b> RD <b>VE</b> CCGD <b>Q</b> L <b>CV</b> WG <b>EC</b> RKATSRGENGTI | 199 |
| <b>HyDkk-3</b>   | 47  | SQE <b>CNA</b> H <b>KAC</b> PE <b>KKY</b> CHL--F-- <b>LC</b> VH <b>CL</b> KEN <b>VACT</b> Q <b>NG</b> Q <b>CC</b> EG <b>Q</b> - <b>CTY</b> G <b>R</b> C <b>KAG</b> VSE <b>GP</b> PTF          | 105 |
| <b>Consensus</b> |     | EC C YC F C C C CC C C G GT   |     |

## Cys-rich domain 2:

|                  |     |   |     |
|------------------|-----|---|-----|
| <b>HDkk-3</b>    | 208 | <b>CD</b> N <b>QR</b> D <b>C</b> --- <b>Q</b> P <b>GL</b> <b>CC</b> A <b>F</b> Q <b>R</b> G <b>L</b> -- <b>L</b> F <b>P</b> V <b>C</b> T <b>PL</b> P <b>VE</b> G <b>EL</b> <b>CH</b> D <b>P</b> A <b>S</b> R <b>LL</b> D <b>LIT</b> W <b>E</b> L <b>EP</b> D <b>G</b> A <b>L</b> D <b>R</b> C--                           | 235 |
| <b>MDkk-3</b>    | 208 | <b>CD</b> N <b>QR</b> D <b>C</b> --- <b>Q</b> P <b>GL</b> <b>CC</b> A <b>F</b> Q <b>R</b> G <b>L</b> -- <b>L</b> F <b>P</b> V <b>C</b> T <b>PL</b> P <b>VE</b> G <b>EL</b> <b>CH</b> D <b>P</b> T <b>S</b> <b>QL</b> L <b>D</b> L <b>IT</b> W <b>E</b> L <b>EP</b> E <b>G</b> A <b>L</b> D <b>R</b> C--                   | 235 |
| <b>GDkk-3</b>    | 200 | <b>CEN</b> Q <b>H</b> D <b>C</b> --- <b>N</b> P <b>G</b> T <b>CC</b> A <b>F</b> Q <b>K</b> E <b>L</b> -- <b>L</b> F <b>P</b> V <b>C</b> T <b>PL</b> P <b>EE</b> G <b>EP</b> <b>CH</b> D <b>PS</b> <b>NR</b> L <b>L</b> N <b>LIT</b> W <b>E</b> L <b>EP</b> D <b>G</b> V <b>L</b> E <b>R</b> C--                           | 257 |
| <b>HyDkk-3</b>   | 106 | <b>CD</b> R <b>H</b> E <b>D</b> C <b>A</b> G <b>E</b> G <b>K</b> A <b>CC</b> V <b>R</b> E <b>P</b> A <b>I</b> N <b>PH</b> I <b>S</b> I <b>C</b> K <b>P</b> L <b>A</b> E <b>N</b> M <b>V</b> C-- <b>G</b> P <b>I</b> N <b>F</b> F <b>R</b> N <b>V</b> Y <b>V</b> G <b>A</b> ---- <b>Q</b> V <b>Q</b> K <b>A</b> C <b>G</b> | 164 |
| <b>Consensus</b> |     | C DC CC C P C P C   |     |

|                  |     |   |     |
|------------------|-----|---|-----|
| <b>HDkk-3</b>    | 236 | <b>PC</b> A <b>S</b> G <b>LL</b> C <b>Q</b> PH-S <b>H</b> S <b>L</b> V <b>Y</b> V <b>C</b> K <b>P</b> T                           | 257 |
| <b>MDkk-3</b>    | 236 | <b>PC</b> A <b>S</b> G <b>LL</b> C <b>Q</b> PH-S <b>H</b> S <b>L</b> V <b>Y</b> M <b>C</b> K <b>P</b> A                           | 257 |
| <b>GDkk-3</b>    | 258 | <b>PC</b> A <b>S</b> G <b>L</b> I <b>C</b> Q <b>P</b> Q <b>S</b> S <b>H</b> S <b>T</b> T <b>S</b> V <b>C</b> E <b>L</b> S         | 280 |
| <b>HyDkk-3</b>   | 165 | <b>P</b> C <b>K</b> Q <b>A</b> L <b>I</b> C <b>K</b> Q <b>V</b> G <b>L</b> F <b>G</b> I <b>H</b> E <b>I</b> C <b>M</b> K <b>E</b> | 187 |
| <b>Consensus</b> |     | PC L C C  |     |

**Fig. 1A, B** *Hydra Dkk-3* cDNA sequence and alignment with most closely related *Dickkopf* genes. **A** Nucleotide sequence and deduced amino acid sequence of *HyDkk-3*. The signal peptide sequence is *underlined*. The predicted endopetidase cleavage side is indicated by an *arrow*. Cysteine-rich domain 1 is shaded in *dark*

*gray*, cysteine-rich domain 2 in *light gray*. Start and stop codons are shown in *bold*. **B** Amino acid sequence comparison of cysteine-rich domain 1 and 2 of *Hydra Dkk-3* with the corresponding regions in human *Dkk-3* (*HDkk-3*), mouse *Dkk-3* (*MDkk-3*), and chicken *Dkk-3* (*GDkk-3*)

searches using the Dkk3-*Gallus* amino acid sequence (Q90839) as the query sequence. The full length cDNA sequence was obtained by combining the available sequence information at <http://ghost.zool.kyoto-u.ac.jp/indexr1.html> and the DOE Joint Genome Institute at <http://genome.jgi-psf.org/ciona4/ciona4.home.html>. The full genomic sequence was found at <http://genome.jgi-psf.org/ciona4/ciona4.home.html>, Annotation/Gene Model ci0100149464.

In addition to the NCBI Database the following databases were used for the in silico search for *Dkk* related genes in protostomes: [http://www.sanger.ac.uk/Projects/C\\_elegans/](http://www.sanger.ac.uk/Projects/C_elegans/) and <http://www.wormbase.org/db/searches/blat> for *C. elegans* and <http://www.fruitfly.org/> for *D. melanogaster*.

## Results and discussion

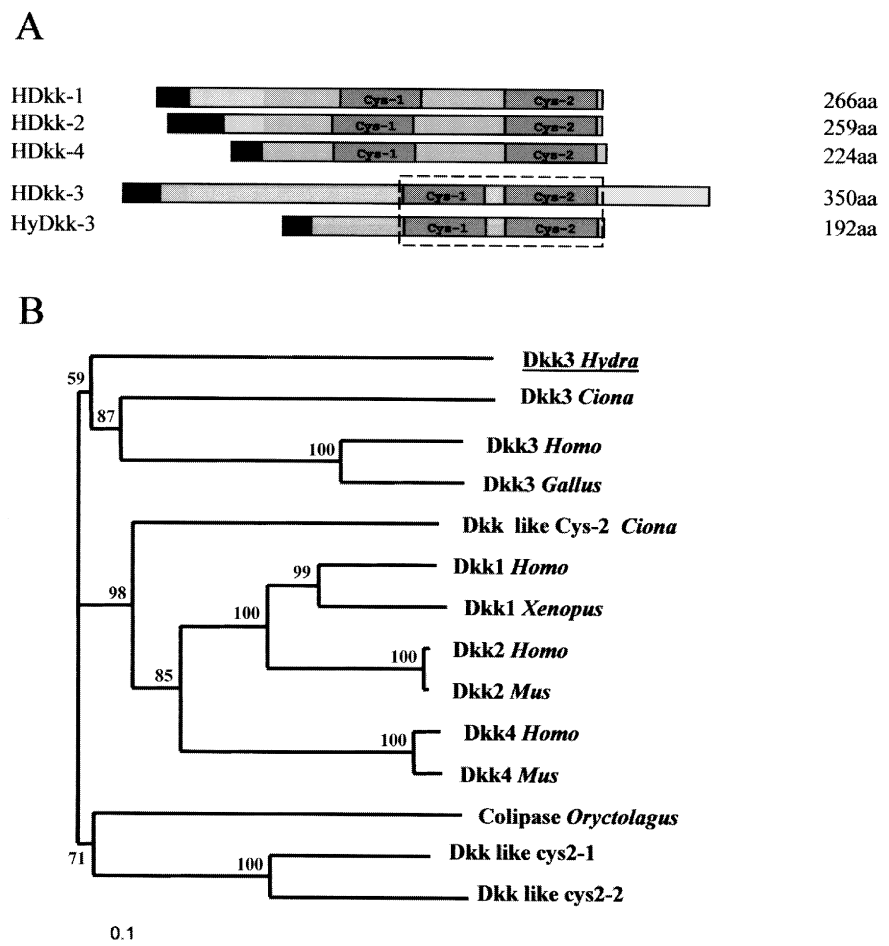
### Isolation and sequence analysis of the *HyDkk-3* cDNA

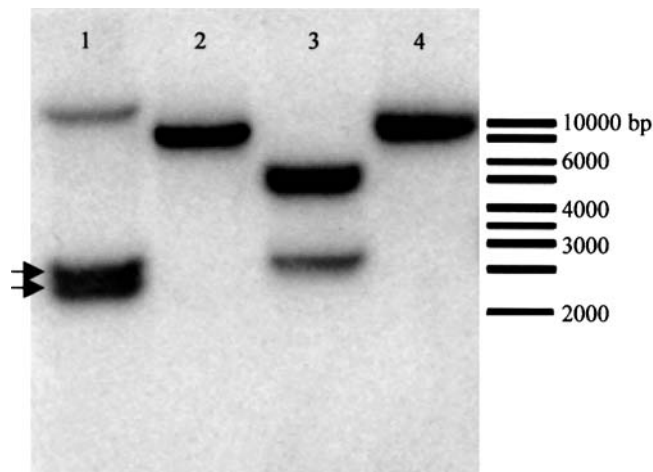
To identify genes involved in HEADY (Lohmann and Bosch 2000) signaling, we used DD PCR screening to examine cDNAs displaying up or down regulation following peptide treatment. One of these was represented in a cDNA which was of 615 bases and predicted to encode part of cysteine rich domain 1 (Cys-1) as well as complete cysteine rich domain 2 (Cys-2) typical for rat Dickkopf 3 (Dkk-3). The full length cDNA sequence was obtained by 5' and 3' RACE. As shown in Fig. 1A, the full length sequence is 882 bases long. The ATG is preceded

by two stop codons and the ORF is terminated by several stop codons followed by a poly(A) tail. The sequence of the 5' UTR with the two stop codons (Fig. 1A) upstream of ATG was confirmed by sequencing the corresponding genomic fragment. The predicted protein has a molecular weight of 21.2 kDa. Hydrophobicity analysis revealed a 17-amino-acid signal peptide at the N terminus which has a defined endopeptidase cleavage site in between amino acid residue 17 and 18.

Blast search comparison of the full length *Hydra* gene indicated the presence of two cysteine-rich domains with highest similarity to chicken *Dickkopf* gene 3 (Fig. 1B) and revealed strong conservation of amino acids known to be of structural and functional relevance. Most prominent among these are a number of conserved cysteine residues described (Brandon and Tooze 1999) as essential for disulfide bond formation (Fig. 1B). Moreover, the linker sequence between the two cysteine-rich domains is 12 amino acids long (Fig. 1A) and, therefore, of identical length to the short linker present in Dkk-3 proteins in vertebrates (Krupnik et al. 1999). In contrast, Dkk-1, 2 and 4 proteins have a conspicuously longer spacer separating the two cysteine domains. Figure 2A shows the structural similarities between *Hydra* Dkk-3 and human Dkk-3 in comparison to Dkk-1, 2 and 4 in human.

**Fig. 2A, B** Evolutionary relationship of *HyDkk-3* and related Dickkopf proteins. **A** Schematic diagram depicting the structural similarities between *Hydra* Dkk-3 (*HyDkk-3*) and human Dkk-3 (*HDkk-3*) in comparison to Dkk-1, 2 and 4 in human (*HDkk-1*, *HDkk-2*, *HDkk-4*). **B** *HyDkk-3* groups together with Dkk-3 of *Ciona*, chicken and man. It is distinct from vertebrate Dkk-1, Dkk-2, and Dkk-4 and from the group of proteins containing only cysteine-rich domain 2 (such as the *Oryctolagus* colipase or Dkk-like cys2-1 and cys2-2 in *Hydra*). Scale bar indicates an evolutionary distance of 0.1 aa substitution per position in the sequence. The numbers at the nodes are an indication of the level of confidence, given in percentage, for the branches as determined by bootstrap analysis.





**Fig. 3** *HyDkk-3* is a single copy gene. Southern blot analysis of *Bcl*I (lane 1), *Eco*RV (lane 2), *Hinc*II (lane 3) and *Xba*I (lane 4) - digested DNA from *Hydra magnipapillata*

The localization of the two cysteine-rich domains is strikingly similar between *Dkk-3* in human and *Hydra*. In comparison to vertebrate *Dkk-3*, the N terminal region of the *Hydra* protein is rather short (Fig. 2A). Phylogenetic tree analysis using the cysteine-rich domain 2 (Fig. 2B) confirmed that the *Hydra* protein groups together with the *Dkk-3* proteins in *Ciona*, man and chicken and is distinct from *Dkk-1*, 2 and 4 proteins. For that reason we have decided to name the *Hydra* gene *HyDkk-3* (AY332609).

#### *HyDkk-3* is a single copy gene

In vertebrates, *Dickkopf* proteins are encoded by at least four related genes (Glinka et al. 1998; Krupnik et al. 1999). Since the *Hydra* genome predates the expansion in gene number that occurred in vertebrates (Collins 1998), it was of interest to determine whether *HyDkk-3* is a single copy gene or member of a gene family. For that purpose we performed Southern blot analysis. Genomic *Hydra* DNA was digested with various restriction endonucleases and probed with a fragment of *HyDkk-3*. Figure 3 shows the hybridization pattern observed. The *Bcl*I digest revealed two distinct fragments hybridizing to the *Dkk-3* probe. The two bands are due to presence of a restriction site for *Bcl*I within the *HyDkk-3* locus (data not shown). The *Eco*RV as well as the *Xba*I digest revealed one distinct fragment hybridizing to the *HyDkk-3* probe. The *Hinc*II digest also revealed one prominent hybridization signal. The weak hybridization signal to an additional 2,500-bp *Hinc*II fragment can be explained by "star activity" of this enzyme. Thus, the restriction pattern shown in Fig. 3 is consistent with the existence of only one *HyDkk-3* sequence in the *Hydra* genome. Identification of *Dkk-3* in *Hydra* lead us to search for other members of the *Dkk* family in *Hydra* using two independent approaches. First, RT PCR cloning gave no evidence that there is an additional *Dkk* gene present in *Hydra* (data

not shown). Second, screening of two *Hydra* EST databases (DDBJ: <http://srs.ddbj.nig.ac.jp/index-e.html>; EBI: <http://srs.ebi.ac.uk>) with vertebrate *Dkk-1* and *Dkk-2* genes resulted in the identification of (1) a single EST containing *HyDkk-3* (tac25c08.y1 at EBI) and (2) the presence of two EST clusters coding for short proteins containing a single cysteine-rich domain homologous to the cysteine-rich domain 2 of *Dkk-1*, *Dkk-4* and *colipase* (for sequence references see Materials and methods). Thus, *Hydra* EST databases provide no evidence for the presence of additional *Dkk* genes containing the two cysteine-rich domains which is the diagnostic feature of the *Dkk* family.

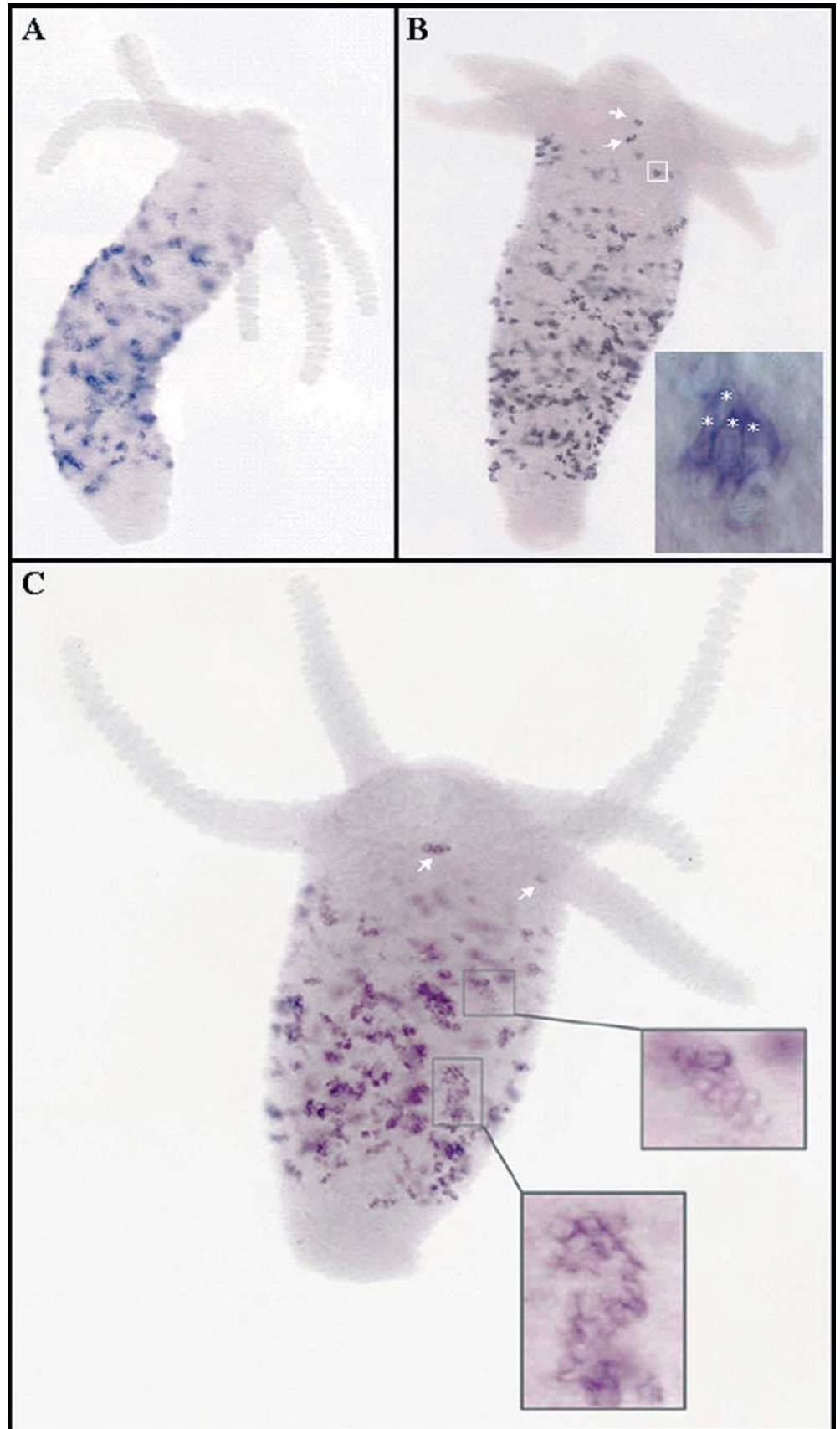
#### Expression of *HyDkk-3* is specific to differentiating nematocytes

The spatio-temporal expression of *HyDkk-3* in *Hydra* polyps was visualized using whole-mount in situ hybridization. As shown in Fig. 4, *Dkk-3* transcripts accumulate in clusters of differentiating nematocytes in the body column. In addition, a small number of *HyDkk-3*-positive nematocytes can be detected in the tentacle formation zone at the base of the tentacles (Fig. 4B, C). These nematocytes, which occur as pairs or small groups but never as large clusters, appear to be migratory nematocytes close to the battery cells in the tentacles where they get incorporated. The conclusion that *HyDkk-3* transcripts appear in nematocytes only at a late differentiation step is supported by the observation that all *HyDkk-3* expressing nematocytes contain fully developed capsules. No expression could be detected in cells at the beginning of nematocyte differentiation nor in head or foot tissue which is free of nematocyte precursors. All four morphological nematocyte types were found to express *HyDkk-3*. Moreover, the same expression pattern could be observed in several strains and species of *Hydra*, including *H. magnipapillata*, *H. vulgaris* (strain AEP) and *H. oligactis* (data not shown). Taken together, the results indicate that *HyDkk-3*, which encodes a secreted signal factor (Fig. 1), is expressed at a late stage during nematocyte differentiation at a time when cells in the clusters start to break-up their cytoplasmic bridges and begin to migrate towards the tentacles. Our data also show that the role of this gene appears to be conserved in different *Hydra* species. With regard to the screening approach used to isolate the gene, *HyDkk-3* does not appear to be dependent on Heady activity (data not shown).

#### In silico search for genes of the *Dickkopf* family outside vertebrates

So far no *Dkk* gene has been identified outside vertebrates (Hino et al. 2003). Stimulated by our identification of a *Dickkopf*-related gene in the basal metazoan *Hydra*, we examined the genome and EST data bases of a number of invertebrates for the presence of *Dkk*. Using a public

**Fig. 4A–C** *HyDkk-3* is expressed in differentiating nematocytes late on in their differentiation pathway. Note that polyps shown in **B** and **C** contain a few *Dkk-3*-expressing nematocytes (arrows) at the base of the tentacles. *HyDkk-3*-expressing nematocytes have fully developed capsules (see, for example, inset to **B**). \* Four *HyDkk-3*-expressing nematocytes with fully developed capsules



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CDkk-3: 149 GQSEWLSEEIITSKSDVKRDRGPWKCSIAFFPCESEDEYCFETQTSSECKECKLEGDQCVDNAECCQEPHANYDHQAMCVFGRG
GDkk-3: 115 TGSTIFSETIITSIKGGENKRNHECLIIDEDCETGKYCQFSTFEYKCPCKTQHTHCSRDRVECCGD-----QLCVWGEC
Consensus: S SE I + ++ +C I C +YC + +C+ CK + C + ECC + +CV+G C

CDkk-3: 229 KLSTRPGAIGTICEVPDDCDAGMCCADTPGYARSLCKPNARVGERCSVEPDYNPSEYIVFE---DGQY-FCPCESDLTCVS
GDkk-3: 188 RKATSARGENGTICENQHDCNPGTCCAFQKELLFPVCTPLPEEGEPCH-DPSNRLNLNLTWELEPDGVLERCPCASGLICQP
Consensus: + +T G GTICE DC+ G CCA +C P GE C +P I +E DG CPC S L C

CDkk-3: 307 RGTNKEDNLQDYNTPRFCMPRLNLDRASTKDKVQ
GDkk-3: 268 QSS-----HSTTSVCELSSNETRKNKEDPLN
Consensus: + + ++T CE+ + +D +

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**Fig. 5** Amino acid sequence comparison of cysteine-rich domain 1 and 2 of *Ciona intestinalis* Dkk-3 (*CDkk-3*) with the corresponding regions in chicken Dkk-3 (*GDkk-3*)

domain search tool (BLAST; Altschul et al. 1990) and the GenBank expressed sequence database (dbEST), we searched for EST clones sharing substantial sequence similarities to known *Dickkopf* genes. *D. melanogaster* and *Caenorhabditis elegans* were chosen, because these protostomes offer completed genome projects and a large number of published ESTs. In addition, we decided to search for *Dkk* in *Ciona intestinalis* because there are more than 240,000 ESTs and a first draft of the *Ciona* genome published. *Ciona* belongs to the earliest branch in the chordate phylum and is considered a key organism for understanding the evolution of vertebrate developmental mechanisms (Corbo et al. 2001).

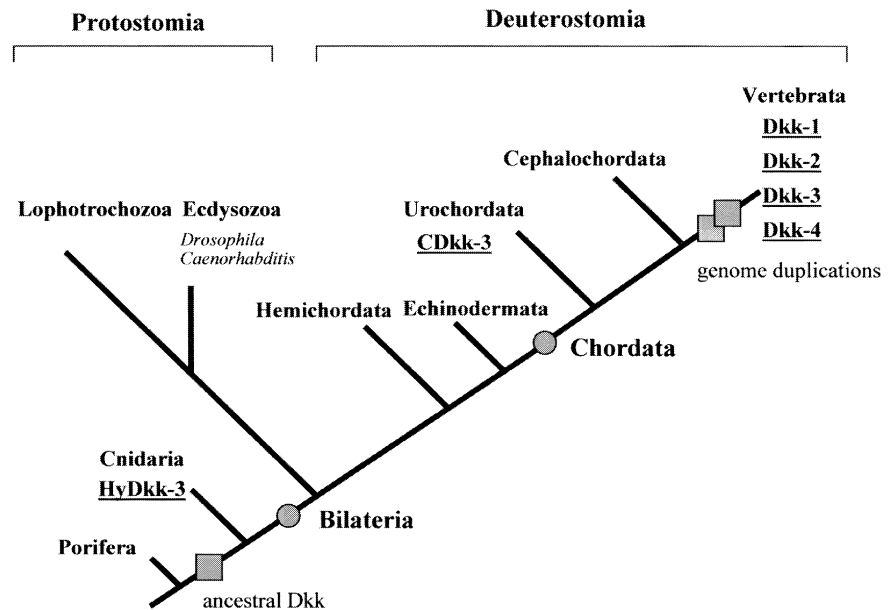
To identify potential *Dickkopf* genes, we searched the genomes and EST sequences of *D. melanogaster*, *Caenorhabditis elegans* and *Ciona intestinalis* for the presence of the two cysteine-rich domains typical of *Dickkopf* genes. The results were surprising. Although EST and genomic sequence comparisons show that *Drosophila* possesses numerous cysteine-rich proteins, there are no obvious matches to the *Dickkopf* sequences. Since the same holds true for the *Caenorhabditis elegans* genome and since in both model organisms genome sequencing is completed, *Dickkopf*-mediated signaling seems not to be conserved in these protostomes. We next searched the *Ciona intestinalis* EST database for the presence of *Dkk* genes. We initially identified eight cDNA clones. Seven clones (ciad068o01, cign052d13, rcilv069e22, cilv004h08, cign028k21, citb042f20, ciad013j05, and cibd068a20) were found to represent the same gene with similarity to human *Dkk-1*, *Dkk-2* and *Dkk-4* (data not shown). After assembling the full length sequence and translating the resulting ORF into a protein sequence, the NCBI database search confirmed that the predicted protein contains a C terminal cysteine-rich domain with similarity to Cys-2 in human *Dkk-1*, *Dkk-2*, and *Dkk-4*. However, the predicted protein had only one of the required two cysteine-rich domains and, moreover, the pattern of the N-terminal-located ten cysteine residues did not fit the characteristic pattern of the *Dickkopf* family (data not shown). We, therefore, do not consider these ESTs to represent *Dkk-1*, *Dkk-2* or *Dkk-4* orthologues.

DNA clone number 8 (cibd068a20) had a high match score *P* value to human *Dkk-3* and revealed the presence of both cysteine-rich domains. When supplemented to a full length clone using the published genome data, the resulting ORF is 1,182 bp long and codes for 394 aa. The homology search in the NCBI database using the full length sequence confirmed the similarity to chicken *Dkk-3*. Alignment of the two cysteine-rich domains of *Ciona* *Dkk-3* with chicken *Dkk-3* is shown in Fig. 5. Interestingly, the comparison revealed not only the *Dickkopf*-typical pattern with the ten characteristically spaced cysteine residues in both domains, but in addition a number of identical amino acids outside the N terminal as well as the C terminal part. Furthermore, the linker sequence between cysteine-rich domain 1 and 2 in the *Ciona* gene consists of 12 aa, which is characteristic of *Dkk-3* proteins and distinguishes them from *Dkk-1*, *Dkk-2* and *Dkk-4*. Therefore, we conclude, that the urochordate *Ciona* contains a *Dickkopf*-related gene, which is most closely related to vertebrate *Dkk-3*, and that there is no evidence for *Dkk-1*, *Dkk-2* and *Dkk-4* in the *Ciona* genome. The apparent conservation of *Dkk-3* in *Hydra*, *Ciona*, and man and the absence of *Dkk-1*, *Dkk-2* and *Dkk-4* outside the vertebrates indicates the intriguing possibility that the four human *Dkk* genes might have originated from a common ancestor gene which is related to *Dkk-3*.

HyDkk-3, a *Dickkopf* protein at the beginning of metazoan evolution

All members of the *Dkk* family are characterized by the presence of Cys-rich domain 1 linked to cysteine-rich domain 2. While the second cysteine-rich domain of mammalian *Dkk-1* and *Dkk-2* was found to inhibit Wnt signaling, the function of the first cysteine-rich domain remains to be shown (Li et al. 2002). Each of these domains contains ten cysteine residues in a highly conserved order. Whereas all members of the *Dickkopf* family have two cysteine-rich domains, a growing number of extracellular proteins have been found in which only a *Dickkopf*-like cysteine-rich domain 2 is

**Fig. 6** The evolution of *Dickkopf* genes. *Dkk-3* was identified in *Hydra* and *Ciona* and is absent in the genomes of *Drosophila* and *Caenorhabditis elegans*. None of the *Dkk-1*, *Dkk-2*, and *Dkk-4* genes of vertebrates have so far been found in invertebrates



present. Examples include colipase and several other secreted proteins of unknown function (Aravind and Koonin 1998; Krupnik et al. 1999). The length of the spacer sequence between cysteine-rich domain 1 and 2 is 50 to 55 amino acids in *Dkk-1*, *Dkk-2* and *Dkk-4*. In all *Dkk-3* proteins isolated so far, the length of this linker region is fixed at 12 amino acids (Krupnik et al. 1999; Monaghan et al. 1999). The *Hydra* *Dkk* gene product described above has a 12-amino-acid linker between the two cysteine-rich domains and is most closely related to chicken *Dkk-3*.

*Dkk-3* has been studied much less extensively than other members of the family and its function is unknown. There is evidence that *Dkk-3*, in contrast to *Dkk-1* and *Dkk-4*, does not modulate Wnt signaling (Krupnik et al. 1999). In vertebrates, *Dkk-3* is expressed in brain and some other tissues, indicating that it might be involved in neuron differentiation or function. In *Hydra*, *HyDkk-3* is expressed in all four types of nematocytes at a late stage of differentiation (Fig. 4). This differentiation step is characterized by changes in morphology and cell behavior that allow extended cell migration of cnidocytes from the gastric region towards the tentacles. Although the function of *HyDkk-3* remains to be elucidated, parallels may exist between *Dkk-3* function in *Hydra* and vertebrates since nematocytes are considered as neuronal sensory cells (Grens et al. 1995; Holstein, personal communication) and since *Dkk-3* in vertebrates is expressed in neuronal tissue.

Finding *Dkk-3* in a basal Eumetazoa gave a reason to search for *Dkk*-related genes in organisms where genome and EST databases are available. While we found no evidence for *Dkk* genes in *D. melanogaster* and *Caenorhabditis elegans*, a *Dkk-3*-related gene could be identified in the *Ciona intestinalis* genome. Beside *Dkk-3*, there is no evidence for the presence of other *Dkk* genes such as *Dkk-1*, *Dkk-2* and *Dkk-4* in *Ciona* (Fig. 6). Most

recently, Satoh and co-workers (Hino et al. 2003) used the *Ciona* genome sequence to identify genes of the Wnt pathway. They also reported identification of a gene, tentatively termed *Ci-dickkopf*, which they propose to be most closely related to human *Dkk-2*. We have re-examined the relation of this gene to members of the *Dickkopf* family and conclude that this gene, due to the presence of only one cysteine-rich domain, does not belong to the *Dkk* family. Thus, since there are four representatives of the *Dickkopf* gene family in vertebrates (see Fig. 6) and since urochordates are the sister group of the vertebrates, it seems likely that the four human *Dkk* genes evolved by gene duplication from a common ancestor gene before the vertebrate radiation approximately 400 million years ago (McLysaght et al. 2002).

The apparent absence of *Dkk* genes in *D. melanogaster* and *Caenorhabditis elegans* was surprising given their presence in *Hydra*, *Ciona*, and vertebrates. Although there have been only a limited number of protostome organisms sequenced, it is tempting to speculate that *Dkk* genes were lost at some point during protostome evolution. Interestingly, a similar observation was reported recently for the *Hydra* *Syk* gene (Steele et al. 1999). Genes of this family of protein tyrosine kinases are absent from the *C. elegans* genome but present in *Hydra* and deuterostomes.

Until recently, genes being absent in *Drosophila* and *Caenorhabditis* but present in mouse and man were thought to have recent origins within the vertebrates. Their identification in *Hydra* indicates that Cnidaria can prove decisive to help to reconstruct the ancestral bilaterian state. Our observations, therefore, underline the importance of studying basal metazoans before drawing any conclusions about the phylogenetic origin of gene families.

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