

# On the origin of the chordate central nervous system: expression of *onecut* in the sea urchin embryo

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**SUMMARY** We identified a transcription factor of the *onecut* class in the sea urchin *Strongylocentrotus purpuratus* that represents an ortholog of the mammalian gene *HNF6*, the founding member of the *onecut* class of proteins. The isolated sea urchin gene, named *SpOnecut*, encodes a protein of 483 amino acids with one cut domain and a homeodomain. Phylogenetic analysis clearly places the sea urchin gene into this family, most closely related to the ascidian *onecut* gene *HNF-6*. Nevertheless, phylogenetic analysis reveals a difficult phylogeny indicating that certain members of the family evolve more rapidly than others and also that the cut domain and homeodomain evolve at a different pace. In fly, worm, ascidian, and teleost fish, the *onecut* genes isolated so far are exclusively expressed in cells of the central nervous system

(CNS), whereas in mammals the two copies of the gene have acquired additional functions in liver and pancreas development. In the sea urchin embryo, expression is first detected in the emerging ciliary band at the late blastula stage. During the gastrula stage, expression is limited to the ciliary band. In the early pluteus stage, *SpOnecut* is expressed at the apical organ and the elongating arms but continues most prominently in the ciliary band. This is the first gene known that exclusively marks the ciliary band and therein the apical organ in a pluteus larva, whereas chordate orthologs execute essential functions in dorsal CNS development. The significance of this finding for the hypothesis that the ciliary bands and apical organs of the hypothetical “dipleurula”-like chordate ancestor and the chordate/vertebrate CNS are of common origin is discussed.

## INTRODUCTION

The three main groups of deuterostomes, the chordates, the echinoderms, and the hemichordates, have been shown to be monophyletic (Turbeville et al. 1994; Wada and Satoh 1994; Bromham and Degnan 1999; Cameron et al. 2000; Peterson and Eernisse 2001; Winchell et al. 2002). Although a wide variety of developmental modes (i.e., direct and indirect development) and life forms (i.e., sessile and free living) exists within deuterostomes, the monophyly indicates that they arose from a common ancestor. Insights into the possible common ancestor of deuterostomes can be gained by studying organisms that are as close as possible to the deuterostome origin and to the divergence of protostomes and deuterostomes. Echinoderms represent such a life form, and the sea urchin pluteus larva is one of the indirect developing forms that are similar to the hemichordate tornaria, dipleurula type larvae, the proposed type of larval form that could be ancestral to all deuterostomes (Nielsen 1999). The evolutionary origin of the chordate/vertebrate central nervous system

(CNS) is one of the mysteries that challenges comparative evolutionary developmental biology. A century ago, Garstang (1894, 1928) formulated the paedomorphosis theory that proposed that the ciliary band of a dipleurula type larva could have moved dorsally and fused during evolution to form the neural tube of the ancestral chordate. Recent studies indicate that the genes that are essential for patterning the chordate CNS are expressed in the ciliary band and/or the apical organ of nonchordate deuterostome larvae. These were studies that showed *otx* expression in the ciliary bands in the larvae of the acorn worm *Ptychodera flava* (Harada et al. 2000) and the sea cucumber *Stichopus japonicus* (Shoguchi et al. 2000) and T-brain expression in the apical organ of *Ptychodera flava* larvae (Tagawa et al. 2000). Furthermore, group B *sox* genes and *NK2.1* were found in the ciliary band and apical organ of *Ptychodera flava* larvae (Taguchi et al. 2002; Takacs et al. 2002).

Here we present further data that could indicate a common evolutionary origin of cells of the dipleurula ciliary band and the apical organ and certain cell types of the chordate

CNS by the cloning and comparative expression analysis of a sea urchin ortholog of the *onecut* transcription factor. In all species analyzed so far, *onecut* transcription factors have a function during the development and differentiation of cells of the central nervous system. In *Drosophila melanogaster*, *Halocynthia roretzi*, and zebrafish, all described *onecut* genes are neural specific (Nguyen et al. 2000; Sasakura and Makabe 2001; Hong et al. 2002). In mammals, *onecut* orthologs were found to be expressed in neural crest cells of the midbrain as well as in the spinal cord and postmitotic neurons in early development and later in retina, spinal cord, and dorsal root ganglia development (Landry et al. 1997; Rausa et al. 1997). Additionally, mammalian *onecut* genes may have acquired functions in liver and pancreas development (Landry et al. 1997; Rausa et al. 1997).

## MATERIALS AND METHODS

### Embryo cultures, treatments, and whole mount in situ hybridization

Sea urchins (*Strongylocentrotus purpuratus*) were induced to spawn by intracoelomic injection of 0.55 M KCl solution. Embryo cultures were grown at 15°C and fixed for whole mount in situ hybridization for 2 h in 2.5% glutaraldehyde in phosphate buffer or in 4% paraformaldehyde in MOPS buffer as described in Arenas-Mena et al. (2000). Sea urchin embryo whole mount in situ hybridization was carried out according to the protocol given by Ransick et al. (1993) with several modifications. First, proteinase K treatment was omitted in our experiments. Second, hybridization and post-hybridization washes were carried out at 65°C. The third post-hybridization wash was performed using  $0.1 \times$  SSC instead of  $1 \times$  SSC. Finally, probe concentration was increased to 1 ng/ $\mu$ l, whereas the anti-digoxigenin (DIG)-AP antibody concentration was reduced to a dilution of 1:3500. Staining was carried out using the BCIP/NBT system (Vector Laboratories Inc., Burlingame, VT, USA) according to the manufacturer's instructions. Antisense DIG-labeled RNA probes were synthesized from linearized plasmid DNA using a DIG-RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. DNase treatment was omitted because it was observed that including the unlabeled linearized DNA in the hybridization significantly reduces background.

A thorough time course for nickel treatment to radialize sea urchin embryos is available for the *Lytechinus variegatus* embryo (Hardin et al. 1992). Using the described protocol (treatment using 500  $\mu$ M nickel chloride, at midblastula to mesenchymal blastula), a uniform radialization of all embryos could not be established in *S. purpuratus*. We found that the sensitive period is slightly earlier in *S. purpuratus* (between very early blastula [12 h] and midblastula [18 h] stage) and that the sensitivity of *S. purpuratus* to nickel chloride is five times higher than for the *L. variegatus* embryo. Therefore, we treated the embryos with 100  $\mu$ M nickel chloride, instead of 500  $\mu$ M. Treatment was started at cleavage stages (after the eggs were definitely activated). The nickel chloride was washed out at 20 h of development to prevent the occurrence of unwanted

variable phenotypes. Zinc treatments to animalize the embryos were performed as described by Nemer (1986).

### Library screens

A partial cDNA clone, having homology to the *onecut* homeobox gene, was identified through *S. purpuratus* EST projects (Poustka et al. 1999, 2003). To isolate a full-length cDNA clone, the original clone was used to screen 81,000 cDNA clones of an arrayed oligo(dT)-primed cDNA library enriched for full-length clones (SMART cDNA Library Construction Kit, BD Biosciences Clontech, Palo Alto, CA, USA) and made from 20-h blastula stage embryos. Two positive clones were identified and sequenced completely by primer walking. Probe labeling, filter hybridization, and image analysis were carried out as described previously (Poustka et al. 1999).

### Sequence analysis

One cut homologs across multiple organisms were identified via Wu-BLAST searches versus the complete genome predicted protein sets of human (version 13.31.1, release 31 March 2003), mouse (version 15.30.1, release 6 May 2003), *C. elegans* (wormpep 97), *D. melanogaster* (version 11.3.1), the zebrafish draft (version 15.2.1, release 2 July 2003, www.ensemble.org), and the JGI Ciona predicted protein set (JGI release 1, www.jgi.com). Protein alignments were generated with ClustalW (Thompson et al. 1994). Neighbor joining (Saitou and Nei 1987) and quartet-based maximum likelihood phylogenetic methods (Schmidt et al. 2002) were used to construct the phylogenetic trees. The distance matrix shown in Table 1 was calculated using the program PROTDIST of the BioEdit package (Hall 1999) from the alignments shown in Figure 2. Accession numbers of used protein sequences are as follows: HsOC-2 NP\_004843; HsHNF6 AAD00826.1; MmHNF6b ENSMUSP00000053288; MmHNF6 NP\_032288; ZFOne cut AAL02365; ZFOne cut2 ENSDARP00000026697; DmOne cut AF169227; HrHNF6 BAB15952; CiOne cut ci0100139609; CeCEH-38 NP\_741018; CeCEH-21 CAD20808.1; CeCEH-39 NP\_508342.1; CeC17H12.9 AF045642.2; SpOne cut AY464698. Abbreviations used are as follows: Hs, *Homo sapiens*; Mm, *Mus musculus*; ZF, *Danio rerio*; Hr, *Halocynthia roretzi*; Ci, *Ciona intestinalis*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sp, *Strongylocentrotus purpuratus*.

The sequence of *SpOne cut* has been submitted to GenBank under accession number AY464698.

## RESULTS

### Cloning and sequence analysis of *SpOne cut*

A partial clone of the putative *SpOne cut* gene was identified in an *S. purpuratus* EST project (Poustka et al. 1999, 2003) and was used to screen an arrayed full-length enriched cDNA library prepared from the blastula stage (unpublished data). Two clones with a 4.4-kb insert were identified and completely sequenced by primer walking. The exact insert size was found to be 4344 bp and revealed a 1449-bp open reading frame encoding a protein of 483 amino acid residues. The predicted

**Table 1. Sequence identity matrices**

Seq →	HsHNF6	MmHNF6	ZFOnecut2	HsOC-2	HrHNF6	CiOnecut	SpOnecut	MmHNF6b	ZFOnecut	DmOnecut	CeCl7H12.9	CeCEH-38	CeCEH-21	CeCEH-39
HsHNF6	1.000	1.000	0.967	0.967	0.891	0.891	0.847	0.913	0.836	0.869	0.826	0.576	0.467	0.467
MmHNF6	—	1.000	0.967	0.967	0.891	0.891	0.847	0.913	0.836	0.869	0.826	0.576	0.467	0.467
ZFOnecut2	—	—	1.000	0.934	0.880	0.880	0.815	0.880	0.804	0.836	0.804	0.565	0.489	0.489
HsOC-2	—	—	—	1.000	0.891	0.891	0.847	0.923	0.826	0.869	0.836	0.576	0.467	0.467
HrHNF6	—	—	—	—	1.000	0.945	0.858	0.869	0.782	0.815	0.826	0.543	0.445	0.445
CiOnecut	—	—	—	—	—	1.000	0.836	0.858	0.793	0.804	0.815	0.543	0.445	0.445
SpOnecut	—	—	—	—	—	—	1.000	0.869	0.760	0.793	0.793	0.532	0.456	0.456
MmHNF6b	—	—	—	—	—	—	—	1.000	0.826	0.858	0.826	0.576	0.467	0.445
ZFOnecut	—	—	—	—	—	—	—	—	1.000	0.804	0.771	0.586	0.489	0.478
DmOnecut	—	—	—	—	—	—	—	—	—	1.000	0.836	0.619	0.467	0.478
CeCl7H12.9	—	—	—	—	—	—	—	—	—	—	1.000	0.586	0.478	0.478
CeCEH-38	—	—	—	—	—	—	—	—	—	—	—	1.000	0.532	0.543
CeCEH-21	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.771
CeCEH-39	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000
<i>A. Cut domain</i>														
HsHNF6	1.000	1.000	0.966	0.883	0.850	0.900	0.916	0.833	0.816	0.816	0.716	0.650	0.433	0.433
MmHNF6	—	1.000	0.966	0.883	0.850	0.900	0.916	0.833	0.816	0.816	0.716	0.650	0.433	0.433
ZFOnecut2	—	—	1.000	0.883	0.850	0.883	0.900	0.816	0.783	0.800	0.700	0.633	0.416	0.416
HsOC-2	—	—	—	1.000	0.866	0.883	0.883	0.850	0.783	0.816	0.716	0.633	0.433	0.433
HrHNF6	—	—	—	—	1.000	0.916	0.866	0.800	0.700	0.833	0.683	0.616	0.433	0.450
CiOnecut	—	—	—	—	—	1.000	0.883	0.816	0.750	0.850	0.733	0.616	0.416	0.433
SpOnecut	—	—	—	—	—	—	1.000	0.833	0.766	0.850	0.700	0.683	0.433	0.433
MmHNF6b	—	—	—	—	—	—	—	1.000	0.800	0.800	0.700	0.616	0.433	0.416
ZFOnecut	—	—	—	—	—	—	—	—	1.000	0.683	0.666	0.583	0.433	0.400
DmOnecut	—	—	—	—	—	—	—	—	—	1.000	0.750	0.683	0.433	0.416
CeCl7H12.9	—	—	—	—	—	—	—	—	—	—	1.000	0.666	0.450	0.416
CeCEH-38	—	—	—	—	—	—	—	—	—	—	—	1.000	0.500	0.450
CeCEH-21	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.616
CeCEH-39	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000
<i>B. Homeodomain</i>														
HsHNF6	1.000	1.000	0.966	0.883	0.850	0.900	0.916	0.833	0.816	0.816	0.716	0.650	0.433	0.433
MmHNF6	—	1.000	0.966	0.883	0.850	0.900	0.916	0.833	0.816	0.816	0.716	0.650	0.433	0.433
ZFOnecut2	—	—	1.000	0.883	0.850	0.883	0.900	0.816	0.783	0.800	0.700	0.633	0.416	0.416
HsOC-2	—	—	—	1.000	0.866	0.883	0.883	0.850	0.783	0.816	0.716	0.633	0.433	0.433
HrHNF6	—	—	—	—	1.000	0.916	0.866	0.800	0.700	0.833	0.683	0.616	0.433	0.450
CiOnecut	—	—	—	—	—	1.000	0.883	0.816	0.750	0.850	0.733	0.616	0.416	0.433
SpOnecut	—	—	—	—	—	—	1.000	0.833	0.766	0.850	0.700	0.683	0.433	0.433
MmHNF6b	—	—	—	—	—	—	—	1.000	0.800	0.800	0.700	0.616	0.433	0.416
ZFOnecut	—	—	—	—	—	—	—	—	1.000	0.683	0.666	0.583	0.433	0.400
DmOnecut	—	—	—	—	—	—	—	—	—	1.000	0.750	0.683	0.433	0.416
CeCl7H12.9	—	—	—	—	—	—	—	—	—	—	1.000	0.666	0.450	0.416
CeCEH-38	—	—	—	—	—	—	—	—	—	—	—	1.000	0.500	0.450
CeCEH-21	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.616
CeCEH-39	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000

Sequence identity matrix of the (A) cut domain and the (B) homeodomain of various *onecut* proteins indicates that the two domains evolve at different rates. As an example the sea urchin homeodomain shows higher similarity to the corresponding domain of human *HNF-6* followed by *ZFonecut2* (90%), whereas the cut domain is more similar to *MmHNF6b* (86.9%) followed by the *HrHNF6*. For GenBank accession numbers of the used sequences and abbreviations, see Materials and Methods.

protein contained a single cut domain and a homeodomain (Fig. 1). The onecut-characteristic phenylalanine and methionine residues at positions 48 and 50, respectively, of the homeodomain (Lemaigre et al. 1996) were also found in the protein. These features led us to the conclusion that this cDNA encodes a onecut class homeodomain protein, which we named *SpOnecut*. Over the whole protein coding region the highest similarity found was to the onecut gene *HNF6* from the ascidian *Halocynthia roretzi* (Sasakura and Makabe 2001).

### Phylogenetic analysis

We identified only a single onecut homolog (ci0100139609) in the ciona genome (*CiOnecut*). In contrast, the draft zebrafish assembly includes two onecut homologs, one previously published (Hong et al. 2002) and a novel one (ENS-DARP00000026697; *ZFOnecut2*). We found no additional *onecut* genes besides the two already published homologs (*HNF6* and *OC-2*; Lemaigre et al. 1996; Jacquemin et al. 1999) in the human genome. Similarly, the mouse proteome includes two onecut homologs, the *HNF6* (Landry et al. 1997; Rausa et al. 1997) and a novel one that we term *MmHNF6b* (ENSMUSP00000053288). *Drosophila melanogaster* has a single onecut homolog (Nguyen et al. 2000) as opposed to several in *C. elegans* (Burglin and Cassata 2002).

Sequence comparison of all onecut homologs showed that the two motifs (the homeodomain and the cut domain) evolve at a different rate within different species (Fig. 2, Table 1). The most stable quartet puzzling and neighbor-joining phylogenetic trees were obtained using the complete coding sequence rather than a partial sequence, including either the homeodomain or the cut domain (Fig. 3). The phylogenetic tree confirms that the identified sea urchin gene belongs to the *onecut* gene family. As one of the two zebrafish onecut orthologs is an outgroup to all vertebrate *onecut* genes, we cannot infer when the *onecut* gene duplications seen in vertebrates happened, that is, whether they were duplicated independently in the respective vertebrate species or in their common ancestor. However, as the single Ciona onecut homolog is an outgroup to all vertebrate *onecut* genes, we can assume that the *onecut* gene duplication is likely to have happened on the vertebrate lineage. In contrast, the multiple *C. elegans* homologs are the result of lineage-specific duplications. Preliminary analysis of the incomplete sea urchin genome sequence indicates only one *onecut* gene in sea urchin.

### *SpOnecut* expression during sea urchin embryogenesis

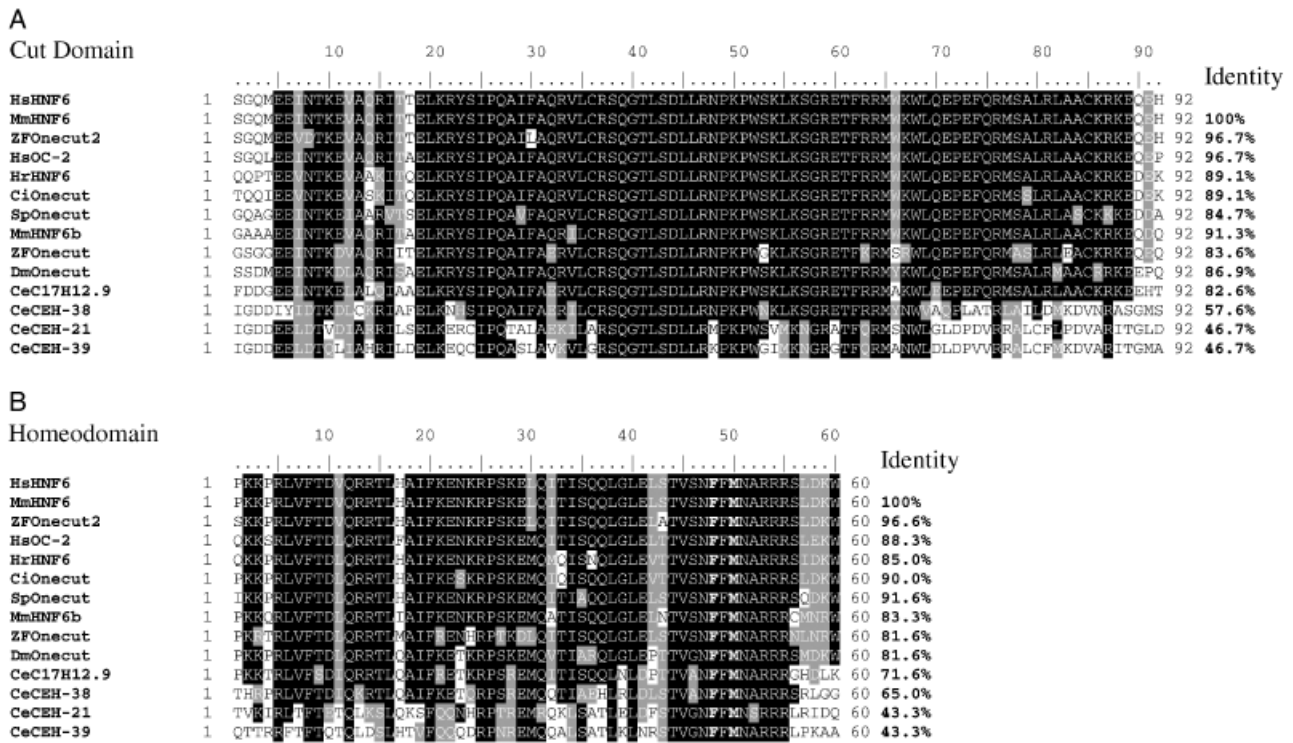
To gain information about temporal and spatial expression patterns of *SpOnecut*, whole mount in situ hybridizations were carried out. Although the original cDNA clone identified originated from an unfertilized egg cDNA library (Poustka

et al. 1999), no significant and localized signals were detected in the embryos before the blastula stage (data not shown). Significant signals above background can be detected in mesenchyme blastula stage embryos at around 30 h of development (Fig. 4, A and B). Here expression becomes apparent in a narrow ring of cells marking the border between the presumptive oral and aboral ectoderm, the future ciliary band of the pluteus larva (Fig. 4A, arrows). As oral–aboral ectoderm differentiation continues and the ciliary band becomes defined, this expression pattern is enhanced. In the late gastrula the *SpOnecut* gene is strongly expressed exclusively in the ciliary band (Fig. 4, C–E) and maintained from here on until the pluteus stage of development. Although the expression is unique to the entire ciliary band at pluteus stage, the expression is strongest in the elongating arms and the apical organ (Fig. 4G, arrow) that carry the long sensory cilia (Fig. 4, F–H). Notably, there is no apparent labeling of any other neural cells of the pluteus larva, apart from putative neural cells in the ciliary band.

Zinc treatment of sea urchin embryos shifts the fate of cells toward the animal (ectodermal) hemisphere of the embryo (Lallier 1955; Timourian 1968; Nemer 1986) at the cost of vegetal (i.e., endomesodermal) tissues and expands the region of cells that carry the long sensory cilia of the sea urchin embryo (also called apical tuft). We have therefore analyzed the expression of *SpOnecut* in zinc-treated embryos to see whether the expression of the gene coextends with this region. We found that this was indeed the case (Fig. 4, I, arrow, and J). There is also expression detected in a band that resembles that of the ciliary band (Fig. 4J, arrow); this is because the treatment with zinc is done only until the midblastula stage and the embryos are then recovering from the treatment and some ectoderm differentiation may become apparent, although endoderm differentiation is vastly blocked.

Treating sea urchin embryos with nickel chloride interferes with the ectoderm differentiation in the second (oral–aboral) embryonic axis and results in a radialization of the embryo that represents an intermediate form of animalization that can be induced by zinc chloride treatment (Lallier 1956; Hardin et al. 1992). In such embryos the oral–aboral ectoderm differentiation is severely disturbed in that the ectoderm is largely pushed to oral fate. Only a small area at the vegetal pole of the embryo remains or is differentiated into aboral ectoderm, whereas endoderm differentiation is largely unaffected. Mesoderm development then is disturbed in that, for example, no pigment cells arise (Hardin et al. 1992). In the radialized embryos *SpOnecut* expression is restricted to a band that localizes around the middle of the embryo and the animal pole (Fig. 4, L and M, arrows) in a stage equivalent to a late pluteus stage (here at 72 h of development) (Fig. 4, L and M). This also illustrates the dissociation of the apical organ and the ciliary band in radialized embryos. The cells stained in the apical organ in fact may represent the four to six serotonergic





**Fig. 2.** Multiple sequence alignments of the (A) cut domain and the (B) homeodomain of *onecut* proteins. The alignment was made using the cut and homeodomain in one file to have the same order of proteins in A and B. Positions with gaps were excluded. The alignments were then edited using Bioedit using a threshold of 20% for identity (black) and similarity shading (gray). The phenylalanine at position 48 and the methionine at position 50 in B are characteristic for the homeodomain of *onecut* proteins and are marked by asterisks. Because the precise extent of the cut domain is unknown, we used the domain region as described by Burglin and Cassata (2002). The percentages of identical residues of various proteins in relation to *HsHNF6* are given at the ends of the alignments. For GenBank accession numbers of the used sequences and abbreviations, see Materials and Methods.

neurons of the apical ganglion, which are the first neurons that appear in the early pluteus (Nakajima et al. 2004).

## DISCUSSION

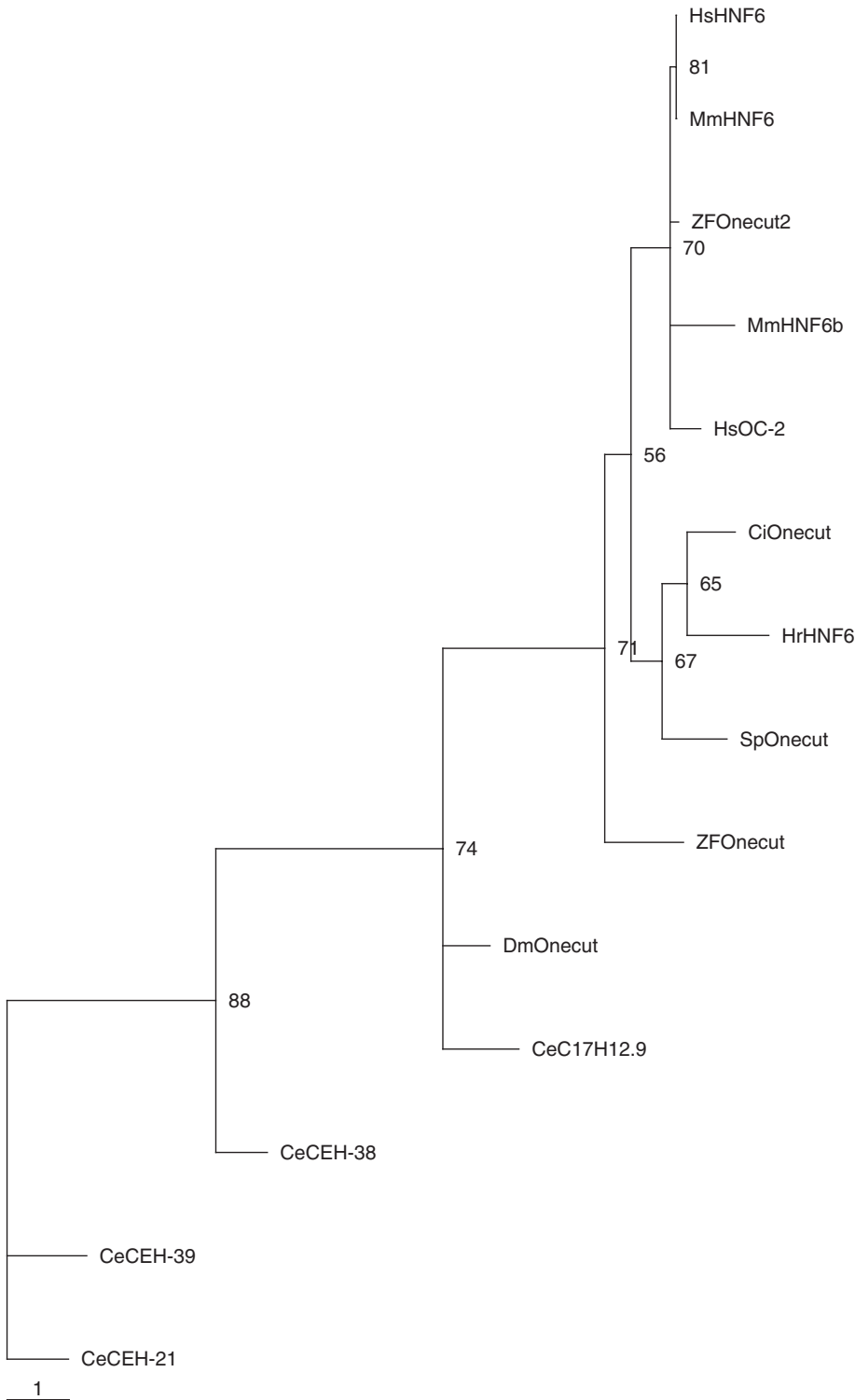
### Phylogeny of *onecut* genes

We cloned and sequenced a gene of the *onecut* gene family in the sea urchin *S. purpuratus*. All members of this gene family share “one” cut domain and a homeodomain, which are highly conserved within the family, although the homeodomain is quite distantly related to homeodomains found in other proteins. Using only the domains that give the family its name, namely the cut and the homeodomain, we were not able to infer stable tree topologies that are in accordance with the species tree, especially for the vertebrate members. We attribute this effect to the variable evolutionary change of the two domains within a single protein and also across species. It was found that on its own the homeodomain has little DNA binding affinity, that DNA binding is mediated mostly by the cut domain, but that the sequence specificity and binding kinetics are greatly influenced by the presence of the homeo-

domain (Lannoy et al. 1998; Catt et al. 1999). This cooperative mode of action of the two domains in *onecut* proteins greatly depends on the *cis*- and *trans*-regulatory environment in cells of any given organism. Because especially *cis*-regulatory changes occur and in fact may be major forces of evolutionary change, different “adaptions” to such changes may appear in different species and cause variable change of the domains. Because different phylogenies are obtained depending on which of the domains are used for the phylogenetic analysis, this may suggest that *onecut* proteins could have been created by domain shuffling (Thornton and DeSalle 2000). Nevertheless, phylogenetic analysis using the entire coding region of 483 amino acids using multiple algorithms (neighbor joining and quartet-based maximum likelihood method) clearly places this gene into the *onecut* family most strongly related to the ascidian *HNF6* gene of *H. roretzi*.

### The ciliary band and the chordate CNS

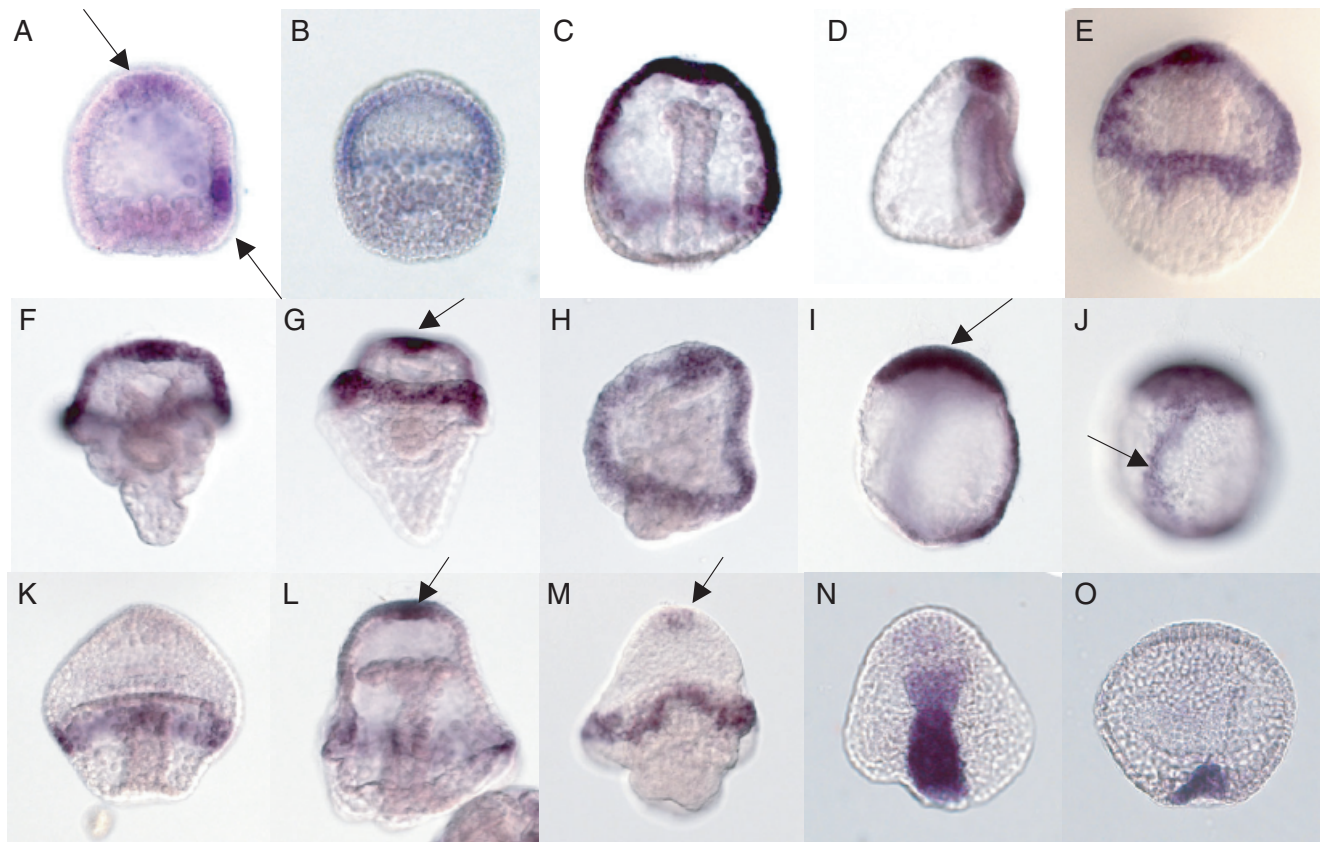
This is the first study that analyzes the expression of *onecut* during early embryogenesis in an indirect (through a primary



**Fig. 3.** Phylogenetic tree of the onecut proteins made using the quartet puzzling method with 1000 bootstraps replicates. Bootstraps values are indicated at the nodes. Sequence distance is indicated at the bottom as the number of substitutions per site. See Materials and Methods for abbreviations and accession numbers.

larva) developing organism. Starting from the early gastrula stage, *SpOnecut* was found expressed specifically in the regions giving rise to the future ciliary band regions and later in the definitive ciliary band of the sea urchin pluteus, including the apical organ. Multiple orthologs of the *onecut* gene family

have been described in ecdysozoans, chordates, and vertebrates. In the ecdysozoan *D. melanogaster* a single *onecut* gene has been found to exist (Nguyen et al. 2000) expressed only in the nervous system: in the CNS during neuroblast formation, in the peripheral nervous system, and also in photoreceptor



**Fig. 4.** Temporal and spatial expression of *SpOnecut*, as revealed by whole mount in situ hybridization. In all panels the animal side of the embryos is located toward the top. (A and B) Late blastula stage embryos. Expression becomes apparent in the future ciliary band. (C–E) Late gastrula stage embryos (45 h) show very strong expression exclusively in the ciliary band. C represents an oral view, B a side view, and E shows an oral view of a flattened embryo to display the band of cells that are stained. (F–H) Pluteus stage embryos (75 h). F and G show side views with different focus planes, H an oral view of a flattened embryo. (I, J, and O) Zinc animalized embryos at 75 h. I and J show the same embryo in different focus planes. O has been hybridized with the gut-specific probe *endo 16* (Ransick et al. 1993) as a control. (K and N) Nickel radialized embryo at 49 h. N has been hybridized with *endo 16* as control. (L and M) 73-h nickel-treated embryos; in M the embryo has been flattened.

cells later in development. This suggests that it may function as a neural specific transcription factor to regulate neural differentiation and possibly plays a role in the maintenance of the neuronal cell phenotype and function (Nguyen et al. 2000). In the chordate *H. roretzi*, throughout embryogenesis the gene is expressed exclusively in neural tissues, namely in the sensory vesicle, the visceral ganglion, and the spinal cord (Sasakura and Makabe 2001). In the teleost fish *Danio rerio*, *onecut* expression is also restricted to neuronal cells, first detected in the trigeminal ganglia neurons at the end of gastrulation. Prominent expression continues in subsets of the neural plate, mid- and hindbrain, and the spinal cord during subsequent development (Hong et al. 2002). In mammals the gene was found to be expressed in neural crest cells of the midbrain, as well as in the spinal cord and postmitotic neurons in early mouse development, and later in retina, spinal cord, and dorsal root ganglia development (Landry et al.

1997; Rausa et al. 1997). Nevertheless, additional functions mainly for liver and pancreas development may have been acquired in mammals (Landry et al. 1997; Rausa et al. 1997). It remains to be determined whether the second zebrafish *onecut* gene is also neuronal specific or whether, similar to mammals, it acquired additional and/or divergent functions in different organs.

Judging from the expression of *onecut* genes exclusively in neuronal cells in ecdysozoans, chordates, teleost fish, and as reported here in the ciliary band of the indirect developing sea urchin pluteus larva, the evolutionary ancient role of this class of genes may be the patterning and differentiation of neural cells, especially of parts of the CNS. Because *onecut* function is also limited to the nervous system in the protostome, *D. melanogaster*, most likely the common ancestor of bilaterians, possessed and used this gene during the development of its nervous system. Because *onecut* orthologs are

expressed in a variety of different types of neuronal cells in protostomes, chordates, and vertebrates, the larval ciliary bands might relate only to certain cell types and not to the whole of the chordate CNS. Interestingly, to date *onecut* gene expression has not been observed in the forebrain of vertebrates. If a homology exists between certain cell types of the CNS of chordates and the apical organ/ciliary band of dipleurula type larva, these data could indicate that such a homology could be restricted to the mid/hindbrain and the spinal cord. The forebrain could then be seen as a vertebrate autopomorphy as suggested by Takacs et al. (2002). In summary, the expression of *SpOneCut* in the ciliary band and the apical organ of a dipleurula type larva, which may represent the type of larva that is most likely the type that is ancestral to all chordates (and possibly bilaterians), could be interpreted to support Garstang's theory that these structures are homologous to the chordate dorsal CNS or at least to certain cell types within the CNS.

There are two main problems with such a view. First, to date there are too few examples known of genes patterning the chordate nervous system that are also used in ciliary band/apical organ morphogenesis of dipleurula type larvae. Second, we do not have any information on the architecture of the regulatory network underlying the formation of the apical organ/ciliary band (a dilemma that in part arises from the first point). Hence, to accept or reject the evolutionary relationship between the nonchordate deuterostome and the chordate nervous system, comparative gene regulatory network analysis for the morphogenesis of the apical organ/ciliary band has to be carried out between at least one dipleurula-like larva and, for example, an annelid trochophore larva. As a second step, regulatory cascades identified then must be compared with the gene networks that function in establishing the chordate nervous system.

If the last common bilaterian ancestor had an apical organ/ciliary band, the network that controls the formation should be found incorporated in the morphogenesis of a (centralized) brain in protostomes and vertebrates. In the case that we are only looking at the simple primary specification and differentiation of neural cells rather than the morphogenesis of the organs that they are deployed within, that should become visible from the analysis of the gene regulatory network. In the latter case the expression of *onecut*, and other such examples, in the apical organ does not tell us whether the apical organ and the ciliary band are homologous with the vertebrate/chordate dorsal hollow nerve cord. Nevertheless, even if the relationship between ciliary bands and the CNS could be proven to be correct by the approach described, this does not exclude that both larval ciliary bands and the CNS already existed in the last common bilaterian ancestor, with the former giving rise to the latter in development.

Views that the last common bilaterian ancestor was not as complex as one could judge from common themes shared

(i.e., in nervous system development or dorsoventral axis specification between arthropods and vertebrates) have been proposed by Erwin and Davidson (2002). Similar views arose recently from the expression analysis of orthologs of genes involved in patterning of the nervous system in chordates in a direct developing hemichordate (Lowe et al. 2003). The trouble with the impressive amount of data generated by Lowe et al. is that the same analysis in an indirect developer might be more informative and that the complexity of the hemichordate nervous system might be underestimated (see also Tautz 2003). Conclusions from this work possibly have to be drawn more carefully. We believe that the paedomorphosis theory of Garstang cannot be the only valid interpretation for the reconstruction of the evolution of chordates because it cannot explain the complexity found in vertebrates. For example, taking principles of engineering and constructional morphology of coeloms into account, as is the core of the "Hydroskelett-Theorie" (Gutmann 1972; Gudo and Grasshoff 2002), it is likely that a worm-like adult is ancestral to chordates and probably bilaterians. In addition, it is likely that this worm-like ancestor had a larval development that was dipleurula-like or at least developed through some form of a primary, ciliary larva (Arendt et al. 2001).

Very recently, Nakajima et al. (2004) showed that the first site of neural cell differentiation in the pluteus lies within the future ciliary band. They also demonstrated that the strongest concentration of nerve cells can indeed be found in the ciliary bands in both echinoid and asteroid larvae, although they also identified a number of previously unknown neural cells outside of the ciliary bands. Most strikingly, they found that the pattern of neural ontogeny seems to be essentially different and implied a derived mode for the pluteus type of development, which in turn could be very significant for tracing nervous systems' origins even further toward chordates. These findings necessitate the analysis of a broader range of larval forms.

Although further single-gene analyses of genes such as the *onecut* gene in both basal protostome and deuterostome larvae must be carried out and will produce further hints pointing in one or the other direction, importantly the analysis described here can provide the starting point for the analysis of the regulatory network underlying the establishment of the pluteus larva type nervous system. In addition, because the ciliary band represents the border between the oral and aboral ectoderm in the sea urchin pluteus, it might also be an essential component in the establishment and maintenance of the oral-aboral axis during sea urchin development and potentially is a key molecule of the respective regulatory network as well.

#### Acknowledgments

We thank two anonymous reviewers for valuable comments. This work was supported by the Max-Planck Gesellschaft zur Förderung der Wissenschaften e.v.

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