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ORIGINAL ARTICLE

Ancient connection between NKL genes and the mesoderm? Insights from *Tlx* expression in a ctenophore

Romain Derelle · Michaël Manuel

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Abstract In recent years, evo–devo studies on non-bilaterian metazoans have improved our understanding of the early evolution of animal body plans. In particular, works on cnidarians suggested that contrary to classical views, the mesoderm originated far before the emergence of the Bilateria. In this context, a synthesis of genomic and functional data concerning the Antennapedia (Antp) superclass of homeobox genes suggested that early in animal evolution, each of the three germ layers was under the control of one cluster of *Antp* genes. In particular, the patterning and differentiation of the mesoderm was under the control of the NKL cluster. The ctenophores stand as a key taxon with respect to such issues because unlike other non-bilaterian phyla, their intermediate germ layer satisfies the strict embryological definition of a mesoderm. For that reason, we investigated the only known member of the NKL group in Ctenophora, a gene previously isolated from *Pleurobrachia* and attributed to the Tlx family. In our analysis of the NKL group, this ctenophore gene branches as the sister-group of bilaterian Tlx genes, but without statistical support. The expression pattern of this gene was revealed by in situ hybridisation in the adult ctenophore. The expression territories of *PpiTlx* are predominantly ectodermal, in two distinct types of ciliated epidermal cells and in one category of gland cells. We also identified a probable endodermal site of expression. Because we failed to detect any mesodermal expression, the results do not provide support to the hypothesis of an ancient functional association between the NKL group and the mesoderm.

Keywords Homeodomain · *Tlx* · Ctenophora · Mesoderm · Evolution

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R. Derelle · M. Manuel
UMR 7138 CNRS UPMC MNHN IRD, Université Pierre et Marie Curie-Paris 6,
Case 05, 7 quai St Bernard,
75005 Paris, France

M. Manuel (✉)
UMR 7138 Systématique, Adaptation, Evolution, CNRS UPMC MNHN IRD, Université Pierre et Marie Curie,
7 quai St Bernard,
75005 Paris, France
e-mail: Michael.Manuel@snv.jussieu.fr

Introduction

In recent years, substantial progress has been made in the identification and characterisation of developmental genes in non-bilaterian animals (sponges, *Trichoplax*, cnidarians and ctenophores). These advances have deeply improved our understanding of the early steps of metazoan evolution (Martindale 2005). Analyses of cnidarian transcriptomes revealed unexpected molecular complexity and provided new data for inferring the evolution of developmentally important genes (Kortschak et al. 2003; Miller et al. 2005; Technau et al. 2005; Jager et al. 2006). The conservation of developmental regulatory systems between non-bilaterians and bilaterians is stimulating new and sometimes provocative hypotheses about major changes in body plans during metazoan evolution. This includes the origin of the nervous system (Galliot et al. 2004), the origin of axial polarity and bilateral symmetry (Hayward et al. 2002; Finnerty et al. 2004; Martindale 2005) and the origin of germ layers, especially of the mesoderm (Spring et al. 2000, 2002; Martindale et al. 2004; Garcia-Fernandez 2005).

Most evo–devo research on non-bilaterian animals has concentrated on cnidarian species. The leading cnidarian models are the freshwater *Hydra* (Wilkins 2000) and the starlet sea anemone *Nematostella vectensis* (Darling et al. 2005) whose genome sequences are completed. A number of developmental gene studies are also available for sponges (e.g., Manuel and Le Parco 2000; Nikko et al. 2001; Larroux et al. 2006) and *Trichoplax* (Martinelli and Spring 2003; Jakob et al. 2004; Hadrys et al. 2005; Monteiro et al. 2006). However, the fourth non-bilaterian phylum, the Ctenophora, remains almost unexplored, with very few reports on gene sequences (Finnerty et al. 1996; Jager et al. 2006) and just one published gene expression pattern (Yamada and Martindale 2002).

The lack of interest in these marine, mostly planktonic, animals is surprising because the ctenophore body plan displays several remarkable features with respect to evo–devo questions (Fig. 1). They have a well-developed subepithelial nerve net and a highly original locomotor system, consisting of many bands of beating ciliated cells (the ctenes or comb plates), longitudinally arranged into eight comb rows (Hernandez-Nicaise 1991; Brusca and Brusca 2003) (Fig. 1). The coordination of movements among the comb rows is under the control of a complex apical organ, which also serves as an organ of orientation and possibly even photoreception (Horridge 1965; Tamm 1982; Hernandez-Nicaise 1974, 1991). The relative disposition of the locomotor system, endodermal canals and prey-catching tentacles (Fig. 1) confers to the ctenophore body plan what can be described as biradial symmetry (i.e., occurrence of only two planes of symmetry). The ctenophore gut is opened at both extremities (the aboral pole bearing a pair of anal pores), contrary to the common claim that only bilaterians have complete guts.

One of the most interesting properties of ctenophores is their true triploblastic condition. Although molecular phylogenies classify them without any doubt as non-bilaterians (possibly as the sister-group of Cnidaria+Bilateria; Manuel et al. 2003; Wallberg et al. 2004; Glenner et al. 2004), ctenophores have true muscle cells independent from epithelia (Hernandez-Nicaise 1991). Furthermore, fate-mapping studies have shown these cells to be derived from endomesodermal micromeres that segregate during the process of gastrulation (Nielsen 1995; Martindale and Henry 1997, 1999). Hence, the ctenophore muscle cell lineage corresponds exactly to the strict embryological definition of a mesoderm (Martindale 2005).

Several recent studies suggested that mesoderm-like elements also occur in cnidarians (Boero et al. 1998; Spring et al. 2000, 2002; Seipel and Schmid 2005, 2006), contrary to the widely admitted view in Zoology textbooks (e.g. Brusca and Brusca 2003) that cnidarians are diploblastic animals. Mesogleal tissues (made of amoeboid cells and/or muscle cells) exist in all three main cnidarian classes, Anthozoa, Hydrozoa and Scyphozoa, and in some cases, they segregate from epithelia quite early during larval or adult body formation (Seipel and Schmid 2006). Expression pattern studies of bilaterian mesodermal genes in cnidarian species (Spring et al. 2000, 2002; Martindale et al. 2004) do not unambiguously argue for the existence of mesoderm in the cnidarian–bilaterian ancestor, but are consistent with this hypothesis. Although homology seems to be the most parsimonious explanation for the shared presence of mesoderm in ctenophores, cnidarians and bilaterians, convergent acquisitions of triploblasty in the various eumetazoan lineages cannot be ruled out.

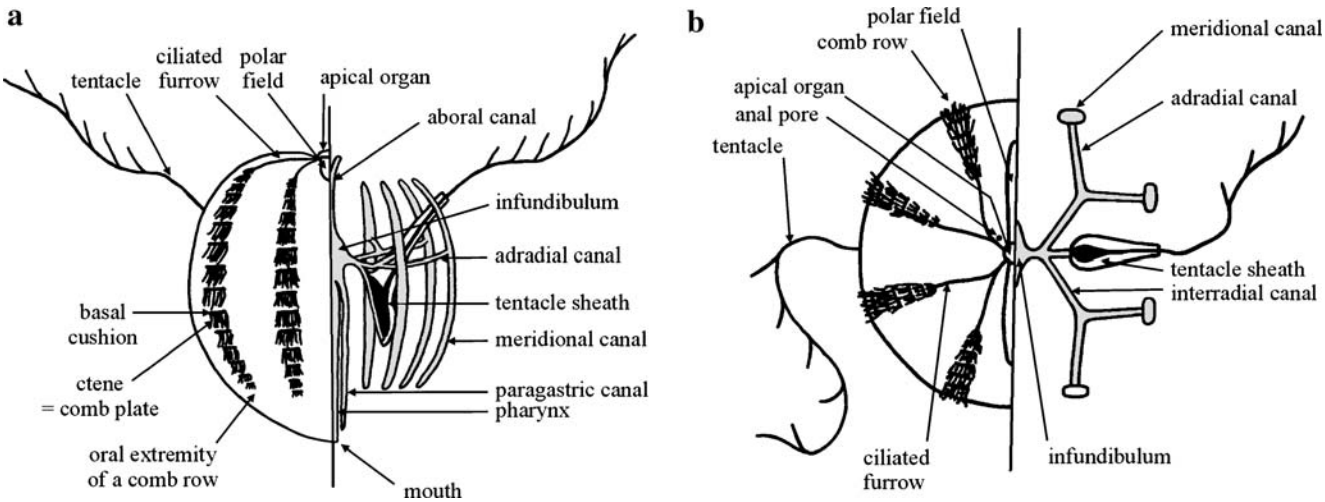

Fig. 1 Schematic representation of the ctenophore anatomy. **a** Adult *Pleurobrachia* in longitudinal view (*oral pole to the bottom*). **b** Adult *Pleurobrachia* in aboral view. In **a** and **b**, the *left half of the drawing* shows the external morphology, and the *right half* figures the internal anatomy. In both drawings, the *vertical line* marks the sagittal plane, perpendicular to the tentacular plane (original drawings). For clarity purpose, the muscle fibers were not figured. They are located below the epidermal and pharyngeal epithelia (parietal muscles) and within the mesoglea (mesogleal fibers). Total length about 1 cm muscle cells

The idea that mesoderm origin predated the evolutionary split between Cnidaria and Bilateria has also received support from recent studies of the evolution of homeobox gene clusters. There is now convincing evidence, from genome comparisons, that all members of the vast Antennapedia superclass of homeobox genes (to which belong, for example, the well-known Hox genes) were originally grouped in a single "Antp megacluster" (Garcia-Fernandez 2005; Kamm and Schierwater 2006; Ryan et al. 2006). A subset of these genes, grouped in a cluster called NKL, share functions in mesodermal patterning and differentiation programs (Jagla et al. 2001; Garcia-Fernandez 2005). The NKL group diversified before the divergence between Cnidaria and Bilateria because cnidarians possess various NKL genes, and these genes are orthologous to the various NKL families present in bilaterian genomes (Kamm and Schierwater 2006; Ryan et al. 2006). Recently, these and other observations gave rise to a provocative theory (Garcia-Fernandez 2005): in a eumetazoan ancestor, the Antp megacluster would have been split into three clusters, each associated with one of the three germ layers, respectively ectoderm, endoderm and mesoderm for the Hox, Para-Hox and NKL clusters (Garcia-Fernandez 2005).

The only known member of the NKL group in ctenophores is the *Tlx* gene of *Pleurobrachia pileus* (Martinelli and Spring 2005). According to the theory, *Tlx* should have a predominantly mesodermal expression in ctenophores. In the present study, we reanalysed the phylogenetic position of this gene within the NK family. Then we looked for its expression pattern in adult ctenophores, to test the hypothesis of an ancient association between NKL genes and the mesoderm.

Materials and methods

Phylogenetic analyses of homeodomain sequences

All homeodomain sequences, including the *PpiTlx* sequence, were recovered from GenBank, with the exception of NKL sequences from *N. vectensis*, which we extracted from the alignment of Kamm and Schierwater (2006). The alignment is provided as Electronic Supplementary Material. Phylogenetic analyses were carried out by heuristic maximum-likelihood (ML) search using the PhyML program (Guindon and Gascuel 2003), with the JTT (Jones et al. 1992) model of amino-acid substitution. A BioNJ tree was used as the input tree to generate the ML tree. A gamma distribution with six discrete categories was used in the ML analyses. The gamma shape parameter and the proportion of invariant sites were optimised during the ML search. Branch support was tested with bootstrapping (300 replicates). We also computed neighbour-joining bootstrap values (500 replicates).

Animal collection and breeding

Adults were collected in Villefranche-sur-Mer (France) during the reproductive season, between March and June. They were kept in 10 l dishes, under continuous water circulation, and fed twice a day with living *Artemia* nauplii and fresh microplankton. Each day, naturally fertilised embryos were collected by filtration soon after spawning time (about 3:00 P.M.) and then raised in Petri dishes in filtered seawater at approximately 18°C.

Preparation of the probe for in situ hybridisation

We performed 3′ rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) (Frohman et al. 1988) from adult cDNA to generate a fragment of sufficient length to synthesise an antisense RNA probe. Before cDNA preparation, adults were left unfed for at least 24 h, to allow prey digestion, and carefully inspected to avoid contamination with other metazoan species. They were then ground to a powder in liquid nitrogen. For extraction of total RNA, we used the "RNeasy Mini Kit" (Qiagen) according to the instructions of the manufacturer. Reverse transcription of cDNA from total RNA extracts (5 to 10 μg) was performed using MMLV-RT (RT-PCR kit, Stratagen) and an oligo-dT primer (5′ GAGAGAAC TAGTCTCGAGT(×18) 3′).

Two specific (non-degenerate) primers were designed from the published sequence of the *Pleurobrachia Tlx* gene and were used as forward primers for PCR amplification. The oligo-dT primer described above was used as the reverse primer. The PCR reaction were performed in a DNA thermal cycler (Biometra) in the presence of specific primer at 1 μM, oligo-dT at 0.2 μM, deoxyribonucleotide triphosphate (dNTP) at 200 μM final, 1× Promega buffer, 1.25 mM MgCl2, and 0.5 U of Taq DNA polymerase (Promega). Samples were amplified for 40 cycles under the following regime: denaturation at 94°C for 1 mn, primer annealing at 60°C for 1 mn and extension for 3 mn at 72°C. Two rounds of semi-nested PCR amplification (with both specific primers) were necessary. PCR products were then gel-purified, quantified, cloned as described in Jager et al. (2003) and sequenced with the "Thermosequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham, Pharmacia) using a fluorescent primer labelled with CY5. Products were analysed using an automatic sequencer (Alf Express, Pharmacia). Our sequences matched exactly with the previously published sequence. Preparation of DIG-labelled RNA sense and antisense probes was done on linearized plasmids using the

RNA DIG-labelling mix (Roche). The length of the probes was 621 nucleotides.

In situ hybridisation

Adults and embryos were fixed in 4% paraformaldehyde in sea water/phosphate buffered saline/tween (PBST) (1:1) for 90 min (adults) or 30 min (embryos) at room temperature, then washed immediately three times for 10 min in PBST (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.5, 0.1% Tween 20) and stored in methanol at −20°C. After stepwise rehydration to PBST and removal of vitelline membranes of embryos, samples were transferred progressively to hybridisation buffer (HB) (5× SSC, 50% formamide, 0.1% tween), prehybridised for 2 h at 65°C in HB with 0.1% dimethyl sulfoxide (DMSO), 50 μg/ml heparin and 100 μg/ml tRNA, then hybridised with DIG-labelled RNA probes overnight at 65°C. Washes were performed in HB at 65°C, then HB/PBST (1:1), then PBST at room temperature. After 30–60 min of incubation in blocking reagent (Roche), 2 h of incubation in anti-DIG Alkaline phosphatase P 1/2000 (Roche) and washes in PBST, colour was developed using BM purple (Boehringer).

The sense probe was used as a negative control (to check for nonspecific fixation of the RNA). We also made a negative control without any RNA probe (to check for endogenous enzymatic activities and nonspecific fixation of the antibody). Both negative controls gave no staining (not shown).

Results

Sequence analysis of the *PpiTlx* homeodomain

In the original paper by Martinelli and Spring (2005), the ctenophore homeodomain called *Tlx* clustered with moderate support (bootstrap value 67%) with Tlx homeodomains from Bilateria, but the analysis was done with the distance neighbour-joining method, and only four sequences (from the Lbx and the NK2 families) were included in addition to Tlx homeodomains. With such a limited gene sampling, it is difficult to draw any conclusion about the orthology relationships of the ctenophore gene within the NKL group, which contains approximately 20 families. As this gene was not included in recent, large-scale analyses of NKL homeodomains (Monteiro et al. 2006; Kamm and Schierwater 2006; Ryan et al. 2006), we deemed it necessary to reanalyse its position.

We used maximum likelihood (ML) to analyse a data set comprising a wide sampling of Antp superclass homeodomain sequences from Bilateria (mainly *Drosophila melanogaster* and *Homo sapiens*), all non-Hox NKL homeodomains from the sea anemone *N. vectensis* (Kamm and Schierwater 2006; Ryan et al. 2006), a representative sampling of published sponge sequences, and the *Tlx* gene from *Pleurobrachia*. All known families of the NKL group are represented in the alignment. The result is shown in Fig. 2 for the NKL group only (the full tree including non-NKL sequences, from which Fig. 2 is extracted, is provided as Electronic Supplementary Material Fig. S1).

In this analysis, the ctenophore gene (*PpiTlx*) clusters without statistical support as a sister-group of the bilaterian Tlx genes. The branch leading to *PpiTlx* is extremely long, with respect to the rest of the Tlx group, indicating high divergence of this ctenophore sequence. However, the position of *PpiTlx* is not likely to be due to long-branch attraction because *PpiTlx* clusters with the shortest branches of this region of the tree (under long-branch attraction, it would group instead with the longest). For that reason, we consider that orthology between *PpiTlx* and bilaterian *Tlx* is likely, although it is not supported by robustness indices. In a recent study of Demox sponge genes, Richelle-Maurer et al. (2006) obtained a similar result, with a different gene sampling. Two sequences from *N. vectensis*, related to the *Tlx* family according to Ryan et al. (2006) (*Nve DQ206223* and *Nve DQ206224* in Fig. 2), group instead with the Lbx family in our tree (Fig. 2), albeit with no statistical support.

Expression pattern of *PpiTlx* in the adult ctenophore

In situ hybridisations were performed with various ontogenetic stages (cleaving embryos, gastrulae, cyddipids and adults), but we could obtain convincing patterns only from adults. The lack of signal from embryonic stages may indicate weak or null expression, or alternatively, may result from technical problems. Therefore, we will not draw any conclusion from this absence of data and will only comment on adult patterns.

In the adult, *PpiTlx* transcripts are detected in four distinct areas (Fig. 3a). We observe a strong staining in ciliated cells forming the border of the polar fields (Fig. 3b and c). Polar fields are elongated areas of ciliated epithelium extending on each side of the apical organ, in the sagittal plane. They are classically interpreted as sensory organs, but their exact function remains enigmatic. In addition, they generate a stream of water that continuously enters the apical organ (Hernandez-Nicaise 1974, 1984). While the entire surface of the polar fields is ciliated (our SEM unpublished observations), only the ciliated cells located at the border express *PpiTlx*. The nearby apical organ does not display any staining (Fig. 3c).

Expression of *PpiTlx* is also detected at both lateral extremities of the basal cushion of each comb plate. The basal cushion corresponds to epidermal cell bodies of the multiciliated cells (called the polster cells) that make up

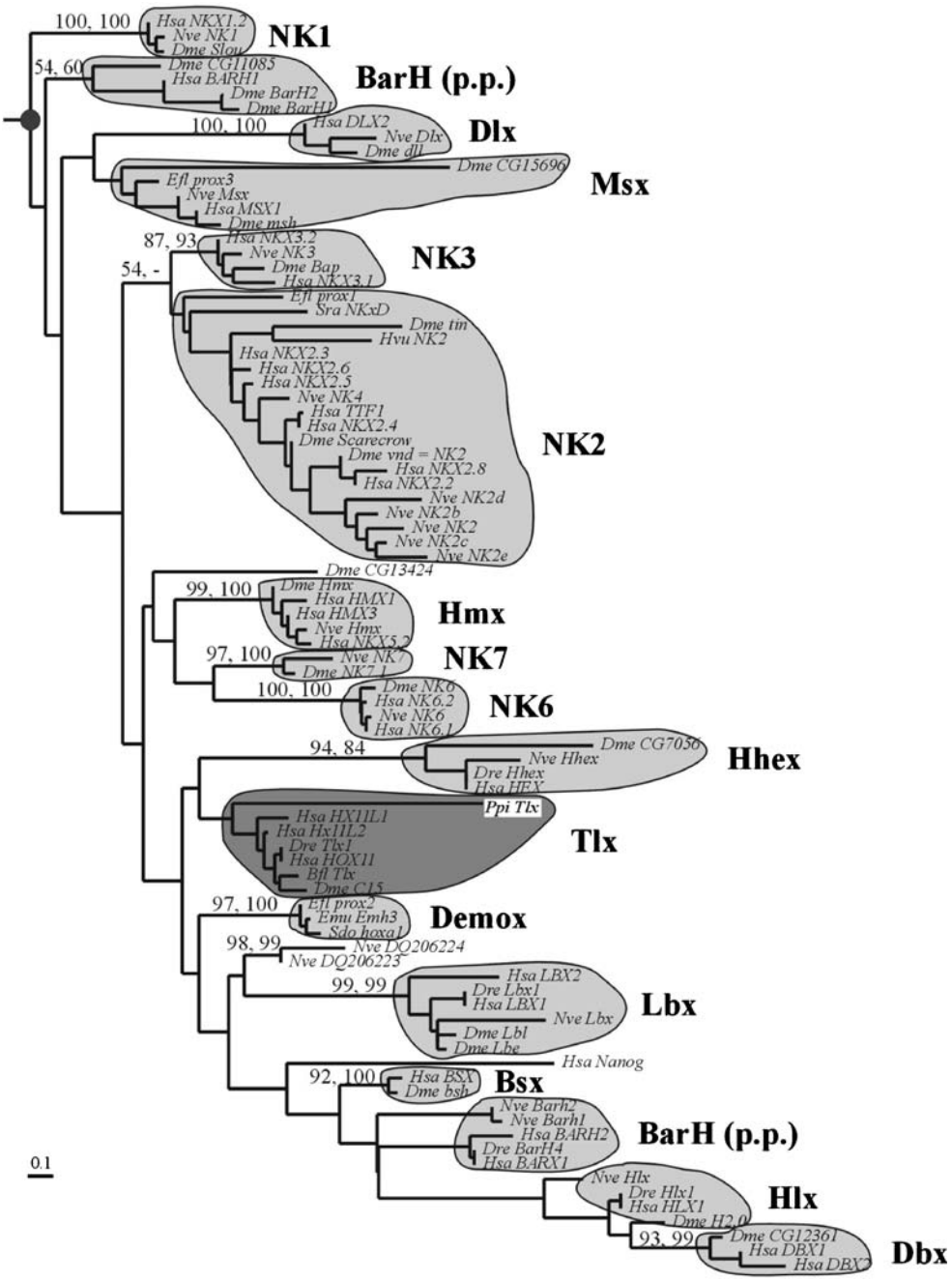

Fig. 2 Maximum likelihood tree of the NKL group. Gene families are *labelled*. Numbers associated with the branches are bootstrap values (only indicated for families and supra-familial relationships and when >50); *first value* ML bootstrap value (300 replicates), *second value* neighbour-joining bootstrap value (500 replicates). *Scale bar* = number of inferred substitutions per position in the sequence

the ctene (Hernandez-Nicaise 1991) (Fig. 3d and e). The stained portion does not correspond to any particular morphological differentiation of the basal cushions (Hernandez-Nicaise 1974, 1991 and personal communication; our own unpublished SEM observations).

A third site of expression is the general epidermis, where the staining is restricted to large scattered cells that are found in higher concentration around the basal cushions of the comb plates (Fig. 3d,e and f). Their size (up to approximately 10 μm in diameter), their rounded shape and the granular aspect of the staining in their cytoplasm (Fig. 3f) suggest they are glandular cells. More precisely, they correspond to the description of spumous gland cells in Hernandez-Nicaise (1991). Although their size is also compatible with a type of sensory cells called hoplocyte (Hernandez-Nicaise 1974, 1991), we failed to observe the conspicuous peg that characterises hoplocytes (Hernandez-Nicaise 1991). A higher concentration of gland cells near the ctenes has not been described previously, but seems plausible from a functional point of view.

The last site of *PpiTlx* expression is at the surface of the meridional canals (Fig. 3g and h). Here, the staining is concentrated in pairs of spots on the external part of each canal. Their distribution is very regular (Fig. 3g). Our

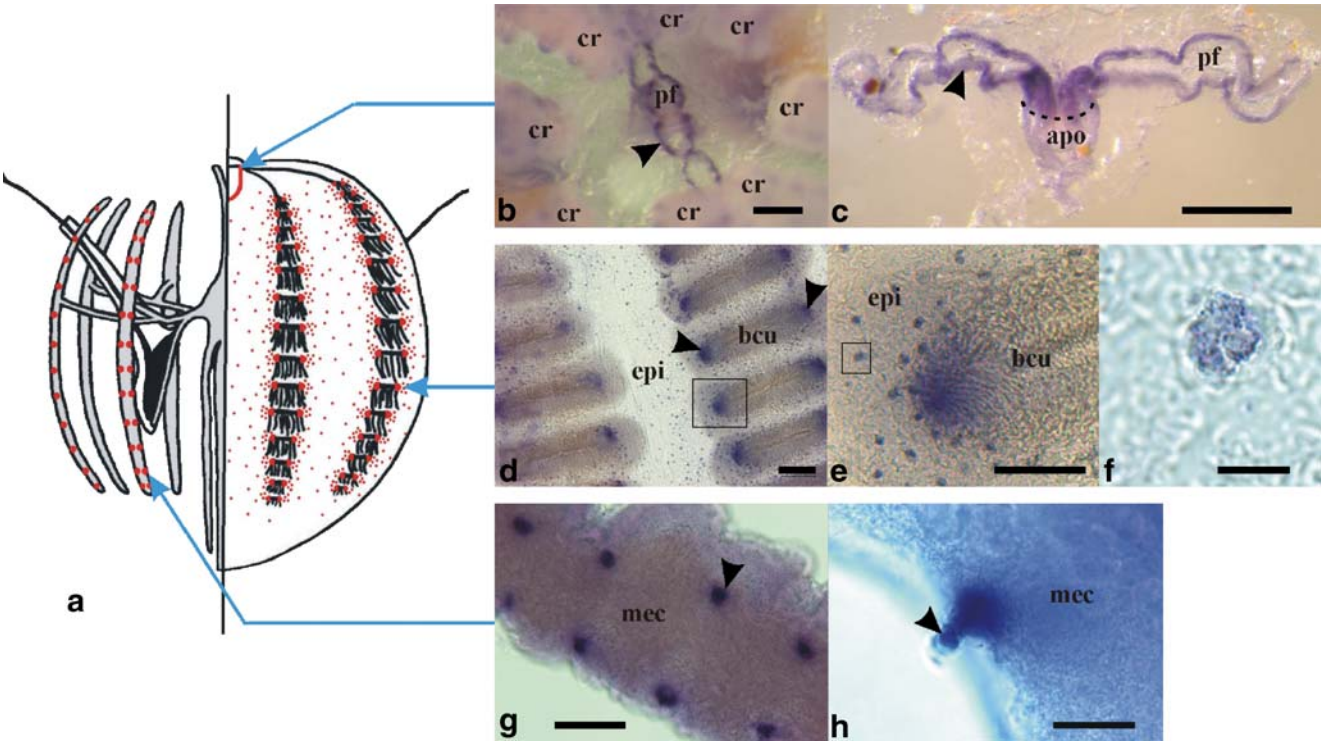

Fig. 3 Expression of *PpiTlx* in the adult *Pleurobrachia*. **a** Drawing summarising the various expression sites. The *blue arrows* indicate the corresponding pictures to the right. **b** Apical view of a whole adult showing expression in the border of the polar fields (*pf*) (*arrowhead*). In the periphery of the picture, the eight comb rows (*cr*) are visible. The apical organ, located towards the middle of the polar field, is not visible here because it is retracted. *Scale bar* = 300 μm. **c** Isolated polar fields (*pf*) and apical organ (*apo*) after microdissection. The *arrowhead* shows the staining in the border of the polar fields. The *dotted line* marks the limit between polar fields and the apical organ. *Scale bar* = 300 m. **d** A portion of epithelium, comprising two adjacent comb rows. The comb plates (made of the cilia of the polster cells) were positioned on the lower side of the preparation, so that they do not hide the basal cushions (*bcu*). The staining can be seen at both extremities of the basal cushions (*arrowheads*) and in isolated cells within the epidermis (*epi*). *Scale bar* = 100 μm. **e** Higher magnification view of the region included in the rectangle in (**d**). *Scale bar* = 50 μm. **f** Higher magnification view of the region included in the rectangle in (**e**), showing one stained spumous gland cell. *Scale bar* = 10 μm. **g** A portion of a meridional canal (*mec*) showing four pairs of stained spots (*arrowhead*). *Scale bar* = 100 μm. **h** Higher magnification of one spot, in profile view, showing the sectioned gonoduct (*arrowhead*). *Scale bar* = 20 μm

detailed examination of whole stained individuals revealed that these pairs of spots alternate with the overlying comb plates. In addition, each spot connects to a thin canal, which is also stained (Fig. 3h). In the light of published anatomical evidence (Hernandez-Nicaise 1991; Martindale and Henry 1997), we identify these canals as the gonoducts, and the spots, as their insertions onto the meridional canals. These spots cannot correspond to the gonads because gonads have a much more extended distribution in the wall of meridional canals.

Discussion

Phylogenetic analysis of the NKL group

Although our tree topology (Fig. 2) places *PpiTlx* as a divergent member of the Tlx family (but without statistical support), we could not identify any Tlx member from the sea anemone *N. vectensis*, consistent with the conclusions of Kamm and Schierwater (2006). In contrast, Ryan et al. (2006) classified two sea anemone sequences (*Nve DQ206223* and *Nve DQ206224*) as Tlx members. In our own analysis (Fig. 2), these sequences do not cluster with *Tlx* genes but with *Lbx* genes (without bootstrap support). Consequently, either these sequences are extremely divergent Tlx orthologues (which we cannot exclude) or a Tlx member was lost in the sea anemone lineage.

With respect to the position of the Tlx family within the NKL group, our analysis (Fig. 2) is not conclusive. Interestingly, we found no evidence for orthology between the demosponge *Demox* (=*Prox2*) genes and *Tlx* genes (Coutinho et al. 2003), as recently shown by Richelle-Maurer et al. (2006), even if such orthology cannot be excluded.

Comparison of *PpiTlx* data with expression and function of *Tlx* genes in the Bilateria

In the Bilateria, *Tlx* genes are involved in a variety of developmental functions (Jagla et al. 2001). Although their

territories of expression include mesodermal derivatives (progenitors of alary muscles in *Drosophila*, Jagla et al. 2001, and the primitive streak in mice, Tang et al. 1998), they are also expressed in endodermal tissues (e.g., splenic primordium in zebrafish, Langenau et al. 2002) and in the peripheral and central nervous systems (in *Drosophila* and in vertebrates, Logan et al. 1998; Jagla et al. 2001; Langenau et al. 2002). In the developing nervous system of vertebrates, *Tlx* genes are involved in the differentiation of particular neuronal types (Logan et al. 1998; Cheng et al. 2004).

The expression pattern of *PpiTlx* in *Pleurobrachia* is not directly comparable with developmental expression patterns of bilaterian orthologues because we only present data for the adult. In addition, the degree of anatomical disparity is so high that it would not make any sense to compare the stained structures in *Pleurobrachia* with particular bilaterian structures where *Tlx* genes are expressed. It is nevertheless possible to formulate general hypotheses about the function of *PpiTlx* in the ctenophore.

Like its bilaterian counterparts (and many, if not most, developmental genes), *PpiTlx* is clearly pleiotropic, with four distinct sites of expression, corresponding to different cell types (ciliated cells of the polar field border, polster cells at the ends of the basal cushion, epidermal gland cells, and epithelial cells associated with gonoducts). The first two types are ciliated sensory cells. Polster cells are not only beating cells, but also mechanoreceptors (Horridge 1965). As neurons and sensory cells have a common evolutionary origin (Galliot et al. 2004), *PpiTlx* expression in these sensory cells may relate to Tlx functions in bilaterian neurons, but the lack of expression of *PpiTlx* in *Pleurobrachia* nerve cells does not lend support to this proposition.

Within each territory, *PpiTlx* expression appears to be restricted to a particular cell subpopulation. In the epidermis, we only saw expression in one particular gland cell type, whereas the epidermis comprises several glandular and sensory cell types, in addition to general epidermal cells. In the same way, it is interesting to note that in the apical region, the staining is restricted to ciliated cells of the polar field border, whereas the area comprises many other ciliated cells, like central cells of the polar field, all cells of the apical organ, and cells belonging to the ciliated furrows. We were also astonished to obtain a localised staining at the end of the basal cushions of the ctenes, although from a morphological and cytological point of view, nothing distinguishes the stained from the unstained polster cells. Overall, these features of *PpiTlx* expression are compatible with an involvement in programs of specification and differentiation of cell subpopulations. Much remains to be done, however, before we can evaluate the degree of functional conservation, at the cellular and molecular level, with bilaterian orthologues.

Were NKL genes primitively involved in mesodermal patterning and differentiation?

The expression of *PpiTlx* in the adult *Pleurobrachia* is predominantly ectodermal, with a probable endodermal site of expression in the gonoducts and their insertion points on the meridional canals (but data are lacking concerning the embryological origin of the gonoduct). On the contrary, we could not detect any signal in mesodermal derivatives, the muscle cells, which are abundant beneath the external epithelium and the pharyngeal epithelium. Hence, *PpiTlx* expression data does not support the hypothesis of a primitive association between homeobox genes of the NKL group and the mesoderm (Jagla et al. 2001; Garcia-Fernandez 2005). Our results do not directly contradict this hypothesis, however, because we only examined one *NKL* gene and only in the adult stage. In addition, the ctenophore mesoderm may not be homologous to mesoderm in other metazoans, and the *Tlx* expression pattern could have been secondarily modified in ctenophores from an ancestral mesodermal territory.

With all these limitations kept in mind, the new ctenophore data adds to an existing array of evidence, which renders the hypothesis vulnerable, as acknowledged by Garcia-Fernandez (2005). For example, in addition to their functions in mesodermal derivatives (reviewed in Jagla et al. 2001 and Garcia-Fernandez 2005), many NKL families are (in some cases predominantly) involved in the development of the nervous system (e.g., in addition to the aforementioned Tlx family: NK2 and NK6 families, McMahon 2000; Lbx family, De Graeve et al. 2004; Hmx family, Wang and Lufkin 2005; NK1 family, Bae et al. 2004; Msx family, Ramos and Robert 2005). Thus, empirical evidence from the Bilateria is equally compelling in favour of a primitive association of NKL genes with the mesoderm or with the neurectoderm (or both). In this respect, the mostly epidermal expression of *PpiTlx* in *Pleurobrachia* seems more consistent with a primitive association with the neurectoderm.

However, the crucial issue is to determine whether or not there is an ancestral functional unity of the NKL cluster, in relation to any particular germ layer. Do we have reasons to think that genes (or multigenic families) need to be functionally associated with particular germ layers or germ layer derivatives? Developmental genes are just elementary tools integrated into developmental processes. Any given developmental process, either cellular (such as apoptosis, cell proliferation, cell adhesion, etc.) or molecular (e.g. a regulatory pathway), may be deployed in a variety of embryological and anatomical contexts (Carroll et al. 2005). Because an embryological germ layer is not an autonomous unit in terms of elementary developmental processes, there may actually not exist marker genes (or

gene families) of germ layers. The proposition that each of the three Antp subcluster is fundamentally linked to one of the three germ layers, although a seductive and stimulating idea, is only weakly supported by empirical evidence and sounds as a manifestation of typological thinking (see Richardson et al. 1999; Jenner 2006).

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