Complementary striped expression patterns of *NK* homeobox genes during segment formation in the annelid *Platynereis*

Alexandra Saudemont [a,1,2], Nicolas Dray [a,1], Bruno Hudry [a,3], Martine Le Gouar [a], Michel Vervoort [a,b], Guillaume Balavoine [a,*]

[a] *Centre de Génétique Moléculaire, C.N.R.S. U.P.R. 2167, 1 avenue de la terrasse, 91198 Gif-sur-Yvette, France*
[b] *UFR de Biologie et Sciences de la Nature, Université Paris 7 — Denis Diderot, 2 place Jussieu, 75251 Paris Cedex 05, France*

Abstract

NK genes are related pan-metazoan homeobox genes. In the fruitfly, *NK* genes are clustered and involved in patterning various mesodermal derivatives during embryogenesis. It was therefore suggested that the *NK* cluster emerged in evolution as an ancestral mesodermal patterning cluster. To test this hypothesis, we cloned and analysed the expression patterns of the homologues of *NK* cluster genes *Msx, NK4, NK3, Lbx, Tlx, NK1* and *NK5* in the marine annelid *Platynereis dumerilii*, a representative of trochozoans, the third great branch of bilaterian animals alongside deuterostomes and ecdysozoans. We found that most of these genes are involved, as they are in the fly, in the specification of distinct mesodermal derivatives, notably subsets of muscle precursors. The expression of the homologue of *NK4/tinman* in the pulsatile dorsal vessel of *Platynereis* strongly supports the hypothesis that the vertebrate heart derived from a dorsal vessel relocated to a ventral position by D/V axis inversion in a chordate ancestor. Additionally and more surprisingly, *NK4, Lbx, Msx, Tlx* and *NK1* orthologues are expressed in complementary sets of stripes in the ectoderm and/or mesoderm of forming segments, suggesting an involvement in the segment formation process. A potentially ancient role of the *NK* cluster genes in segment formation, unsuspected from vertebrate and fruitfly studies so far, now deserves to be investigated in other bilaterian species, especially non-insect arthropods and onychophorans.
© 2008 Elsevier Inc. All rights reserved.

Keywords: Annelid; *Platynereis*; Segmentation; *NK* homeobox genes; *tinman*; Bilaterian evolution; Posterior growth

Introduction

Reconstituting the body plan and major developmental characteristics of the last common ancestor of bilaterian organisms (often called *Urbilateria*) is one of the major challenges of comparative developmental biology. In recent years, based on a number of genetic similarities mostly shared by insects and vertebrates, it has been proposed that *Urbilateria* was a relatively complex annelid-like organism with a differentiated anterior–posterior axis, a digestive tract, a condensed nervous system with a brain and nerve cords, a segmented trunk and appendages (Pennisi and Roush, 1997, Veraksa et al., 2000). One of the key debates is on the question of the origin of metameric segmentation, which may have either one single origin in the bilaterian ancestral lineage (assuming multiple losses in the evolution of extant bilaterian phyla), or, at the opposite end of the hypotheses spectrum, three different origins corresponding to the lineages of the three most overtly segmented phyla (arthropods, annelids and vertebrates) (reviews: Davis and Patel, 1999; Balavoine and Adoutte, 2003; Patel, 2003; Seaver, 2003; Peel and Akam, 2003; Minelli and Fusco, 2004; Tautz, 2004; Damen, 2007). Although morphogenetic and genetic processes of segment formation

---

are known to be very dissimilar between the most well known models in developmental biology, *Drosophila* on one side and the vertebrates on the other side, intriguing similarities have been uncovered between distant phyla that are difficult to explain by mere coincidence. These include the role of *Notch/Delta/hairy/enhancer of split* genes, shared in vertebrate somitogenesis (review: Rida et al., 2004) and in segment formation in a spider (Stollewerk et al., 2003; Schoppmeier and Damen, 2005), and the likely involvement of *engrailed* and *wingless* genes in segment formation in an annelid (Prud'homme et al., 2003), similar to arthropods. In addition to metamerism, it has been proposed that *Urbilateria* would have been a coelomate animal equipped with a blood circulatory system (Hartenstein and Mandal, 2006). A coelomic cavity, blood vessels and a blood pump (or "heart-like organ") are indeed present in most medium- to large-sized bilaterians but usually absent in small-sized species. The roles of the gene *tinman* (*tin* or *NK4*) in the formation of the fly dorsal pulsatile vessel (Bodmer, 1993) and of its vertebrate orthologues in the ontogenesis of the heart (Harvey, 1996) suggest that *Urbilateria* already had a heart-like organ. *tin* is part of a large sub-family of

related homeobox genes, the NKL genes (Pollard and Holland, 2000), encoding homeodomain transcription regulators. Some of these genes are found in a chromosomal cluster in insect genomes, the *NK* cluster (Luke et al., 2003) that originated early in metazoan evolution (Larroux et al., 2007) but has been dispersed in chordates. In fly, all of these genes are involved in patterning some mesodermal derivatives, including the dorsal vessel, the visceral mesoderm and various somatic muscles (Jagla et al., 2001). It has been suggested that this cluster might represent an ancient mesodermal homeobox cluster (alongside a fundamentally ectodermal Hox cluster and an endodermal ParaHox cluster) in which chromosomal organization was selectively retained because of a common regulation. However, the study of the vertebrate orthologues of *NK* cluster genes gives only limited support to this idea since their mesodermal functions are only partially similar to those of their fly counterparts (Jagla et al., 2001).

In order to investigate further the ancestral function of the *NK* cluster, we cloned and analysed the expression patterns of the orthologues of seven genes (*Msx/drop*, *NK4/tinman*, *NK3/bagpipe*, *Lbx/ladybird*, *Tlx/C15/Clawless*, *NK1/Slouch/*
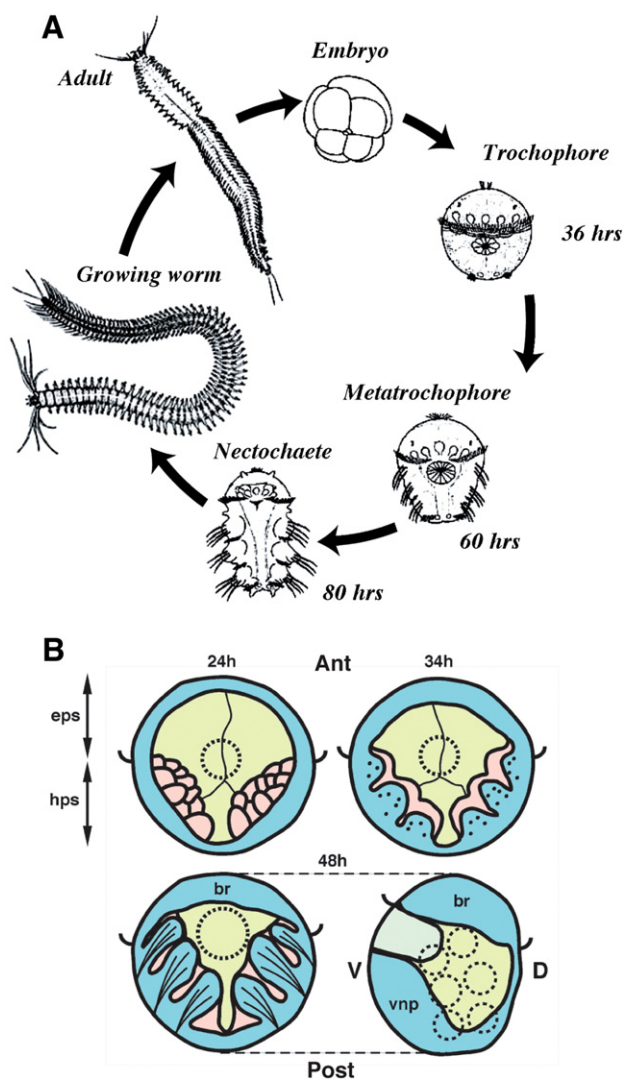

Fig. 1. (A) The life cycle of *Platynereis* (adapted from Dorresteijn et al., 1993) passes through a microscopic free-swimming stage, the trochophore larva. This spherical larva propelled by an equatorial ring of ciliated cells, the prototroch, is lecithotrophic. Apart from a number of specialized larval structures lost at metamorphosis, the *Platynereis* trochophore is nothing more than a swimming embryo in which the organs of the little worm start to differentiate. This involves anterior–posterior elongation, appendage (parapodia) outgrowth, formation of a head to give a three-segment little worm. After 10 days, the 3-segment worm starts to feed. Additional segments proliferate sequentially from a sub-terminal posterior growth zone (de Rosa et al., 2005) for most of the animal life. As the worm trunk is made of identical segments, the process of organogenesis continues for as long as posterior growth proceeds. Therefore the genetic regulation of organogenesis can (and must) be studied during posterior growth as well as during embryogenesis. This process of juvenile posterior growth is quite slow, which is inconvenient when studying dynamic gene expression patterns. Fortunately, we have a way to accelerate segment production considerably: as most annelids, *Platynereis* is capable of caudal regeneration after an amputation of the posterior half of the trunk. The regeneration blastema produces a new anus-bearing terminal piece (pygidium), a new segment addition zone and segments are then added at about a 5-fold accelerated rate (2/day approximately). (B) The development of *Platynereis* during the trochophore stage. Embryos are synchronous: the stages are named with the post-fertilization time (hours post-fertilization, hpf). The three first views are schematic frontal sections in three different stages. The last one is a sagittal section of the 48 hpf larva. Ectoderm is in blue, mesoderm in pink and endoderm in green. Anterior (or apical) is up. Posterior is down. eps: episphere above the prototroch that will give rise to most of head; hps: the hyposphere that gives rise to the trunk tissues. In the 24 hpf trochophore, two paired mesodermal bands have started to proliferate. At 34 hpf, the anlagen of the parapodia are appearing in the form of setal sacs, small invagination of ectodermal cells that will first give rise to the chaetoblasts, the cells producing the bristles or chaetae. There are six setal sacs on each lateral side (two per parapodium). At 48 hpf, the setal sacs have grown deeper and the first setae are being produced. The mesodermal bands are pushed by the growth of setal sacs and mesodermal cells ultimately surround each of the setal sacs, giving notably the parapodial musculature. The ectodermal tissue of the episphere thickens and gives rise to two lateral brain anlagen (br). On the ventral side, the ectodermal covering also thickens to give the ventral neural plate (vnp), precursor of the ventral nerve cord. In the three first pictures, the dotted circle represents the position of the ventral stomodeum. In the last picture, the dotted circles show the position of the left side setal sacs.

S59 and *NK5/Hmx*) in the marine nereid annelid *Platynereis dumerilii*. Nereid annelids are particularly well suited for this study because they present most of the anatomic characteristics suggested above for *Urbilateria*. *Platynereis* is coelomate, segmented and shows vascular, coelomic and muscular systems that are organized metamerically. Additionally, annelids are trochozoans (or lophotrochozoans), the third great phylogenetic branch of bilaterians with deuterostomes (including chordates) and ecdysozoans (including arthropods) (Adoutte et al., 2000). The phylogenetic position of *Platynereis* makes it a crucial model to understand better the evolutionary history of *NK* genes. Strikingly, *Platynereis* shares with vertebrates a remarkably ancestral gene structure (Raible et al., 2005). The formation of trunk segments (and therefore organogenesis as well) occurs in two phases in *Platynereis*. The three first anterior segments are formed during

trochophore development and all the other segments are produced by a posterior growth zone during most of the life of the worm (Fig. 1). For each gene, we therefore compared the expression patterns during embryonic/larval development and juvenile posterior growth (studied after caudal regeneration, see Fig. 1 legend). The expression patterns suggest multiple functions of the *NK* cluster genes in *Platynereis*, including the patterning of mesodermal organs but also more unexpectedly the patterning of segments.

Results

For each *NK* gene considered, we found a single clear orthologue (Fig. 2 and sequence alignments in Supplementary information). This does not mean that other orthologues do not exist in the *Platynereis* genome. However, duplicated pan-
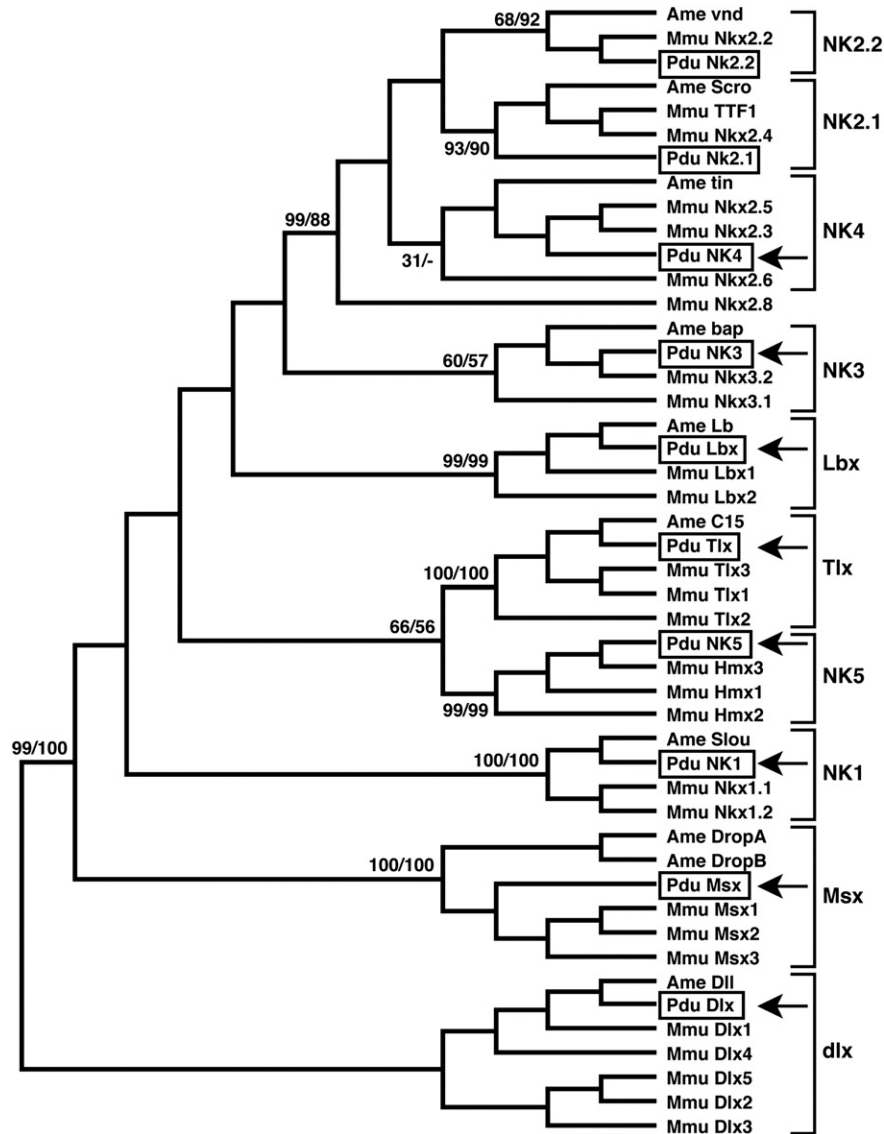

Fig. 2. Phylogenetic NJ tree of the *NK* genes rooted on the closely related *Dlx* genes. Pdu: *Platynereis dumerilii*, Mmu: *Mus musculus*, Ame: *Apis mellifera*. Node values are the percentages for non-parametric bootstrap, when bee genes (left value) or fruitfly genes (right value) are taken as representatives of the insects. The *NK4* orthology group is poorly supported because of the fast-evolving insect *tinman* sequences. Arrows show the genes of which expression patterns are described in this article.

bilaterian genes are known to be remarkably rare in *Platynereis* (Raible et al., 2005).

We performed whole mount in situ hybridizations (WMISH) for each *NK* gene at various stages of embryogenesis, starting from 24 hpf (i.e. early swimming trochophore, after the completion of gastrulation) to 72 hpf (larva with three pairs of segmental appendages called parapodia), complemented in some cases by additional stages of interest. Results from a selection of stages are shown in Fig. 3. We also studied the expression of the *NK* genes during juvenile growth by performing WMISH on regenerated posterior parts in juveniles (Fig. 4).

Pdu–NK4, Pdu–Lbx and Pdu–Msx are expressed in complementary ectodermal stripes

These three genes turn on in the ectoderm in 4 sets of stripes clearly visible at 30–34 hpf, well before overt segmentation (Figs. 3G, H, L, M, Q, R). Expression of all three includes neuronal precursors ventrally, with the *Lbx* and *NK4* domains extending more ventrally than the *Msx* domain. The three anterior-most stripes of each of the three genes are interrupted dorsally at the level of a thin ectoderm that covers the yolk and the first stripe appears shorter and fainter. *Msx* stripes are broader and fuzzier than those for *NK4* and *Lbx*. There are also important differences in the timespan of deployment of the gene expressions. *Msx* stripes are quite transient as they fade very shortly after 30 hpf but they remain visible laterally at least until 48 hpf, as patches in the parapodial area (Fig. 3S). *NK4* stripes also fade from 40 hpf onward (not shown) and only a long period of staining still reveals them at 48 hpf (Fig. 3I), when the segments are about to take shape. The four paired *Lbx* stripes persist in late trochophore stages (Fig. 3N) and 72 hpf larvae (Fig. 3O), fusing over the ventral midline. As indicated by the relative position of setal sacs, the *Lbx* stripes cover the posterior part of each forming segment, only interrupted by small ventral bilateral gaps at the level where the prospective ventral nerve cord (VNC) contacts the parapodial ectoderm.

During posterior segment addition, *Msx*, *NK4* and *Lbx* are also expressed in ectodermal stripes very similar to the ones seen during embryonic/larval development (Figs. 4A–D). These stripes appear very early in the segment addition zone, well before any trace of segmentation is visible but in contrast to embryogenesis, they persist in differentiating segments. We can thus follow their position within each segment, especially on tissue sections (Figs. 4H–J). Thin *NK4* stripes are located in cells immediately posterior to the segmental groove between adjacent contiguous segments. Thicker ectodermal *Lbx* stripes appear on the opposite side of the segmental groove. In contrast to *NK4*, *Lbx* stripes grow with the segment anlagen, covering the posterior part, including the ventral neuroectoderm and the posterior part of each forming parapodium. *Msx* is expressed in thick stripes covering the middle part of each forming segment, including the parapodia, but not the ventral neuroectoderm.

Using double probe WMISH and confocal microscopy, we analyzed the respective positions of the ectodermal stripes of expression of the three *NK* genes as well as the homologues of *engrailed* (*Pdu–en*) and *distal-less* (*Pdu–Dlx*) during embry-

ogenesis (Fig. 5). *Pdu–Dlx* is expressed in ectodermal cells that will give rise to the parapodia epidermis and is a marker of the medial part of segments (Figs. 5A, C). *Pdu–en* (Prud'homme et al., 2003) is expressed in stripes in the anterior-most cells of future segments (Fig. 5D). A first series of double WMISH on late trochophore stages (30 or 34 hpf) with *Dlx* as the reference gene show that *NK4*, *en* and *Lbx* are all expressed in stripes outside of the parapodial field, at the segmental margins (Figs. 5E, G, H). *Lbx* at 48 hpf is expressed in broader stripes that overlap on the posterior part of each parapodial field (Figure S2A, white arrowheads). *Msx* stripes broadly overlap with the *Dlx* expression at 30 hpf (Fig. 5F) and are thus mid-segmental in position. The expression domains of *en* and *NK4* during posterior growth are located in a ring of cells at the segment margin, that appear to be the anterior most ectodermal cells in each appearing segment anlagen. This raises the possibility that these two genes are expressed in the same cells and this is indeed what we observe in embryonic double WMISH in lateral ectoderm (Fig. 5J, J′). At the level of the ventral neuroectoderm, the situation appears more complex as the stripes of *en* and *NK4* become discontinuous and do not overlap. The *NK4* stripes appear to be directly abutting posteriorly the *Lbx* stripes in larvae (Figs. 5K, K′) in perfect correspondence with their respective positions during posterior growth on each side of the segmental groove.

Pdu–Msx, Pdu–Lbx, Pdu–Tlx and Pdu–NK1 are expressed in pre-segmental mesodermal stripes

These mesodermal stripes are clearly seen only during posterior growth, not during embryonic/larval development. Similar to the ectodermal stripes, they appear when segmentation is not yet visible but persist in growing and differentiating segments. As the annelid mesoderm is organised in segmental units that we can call somites (by analogy with chordates), we were able to locate on tissue sections the respective position of these stripes. *Lbx* mesodermal stripes appear roughly in frame with the ectodermal stripes in wholemount specimens but they are shown on tissue sections to belong to the adjacent posterior segments (Fig. 4I, red arrowheads), just posterior to the somite boundary (the septum). The *Msx* mesodermal stripes cover the median part of the somites on the body wall side (somatic mesoderm) (Fig. 4J, red arrowheads). The *Tlx* stripes represent the posterior-most cells of each somite on the somatic side (Fig. 4K, red arrowheads) and they extend inside the posterior part of each forming parapodium. Last, the *NK1* stripes appear to cover the anterior-somatic part of somites (Fig. 4L, red arrowheads), similar to *Lbx*. These somitic mesodermal stripes for all four genes extend in the lateral sides of the trunk but are interrupted ventrally and dorsally at the level of the unsegmented ventral and dorsal vessels, respectively.

Pdu–NK4, Pdu–Lbx, Pdu–Msx, Pdu–NK3, Pdu–Tlx and Pdu–NK1 are expressed in distinct mesodermal organ precursors

These patterns are clearly distinct from the mesodermal stripes described above. *NK4* is strongly expressed in cells that
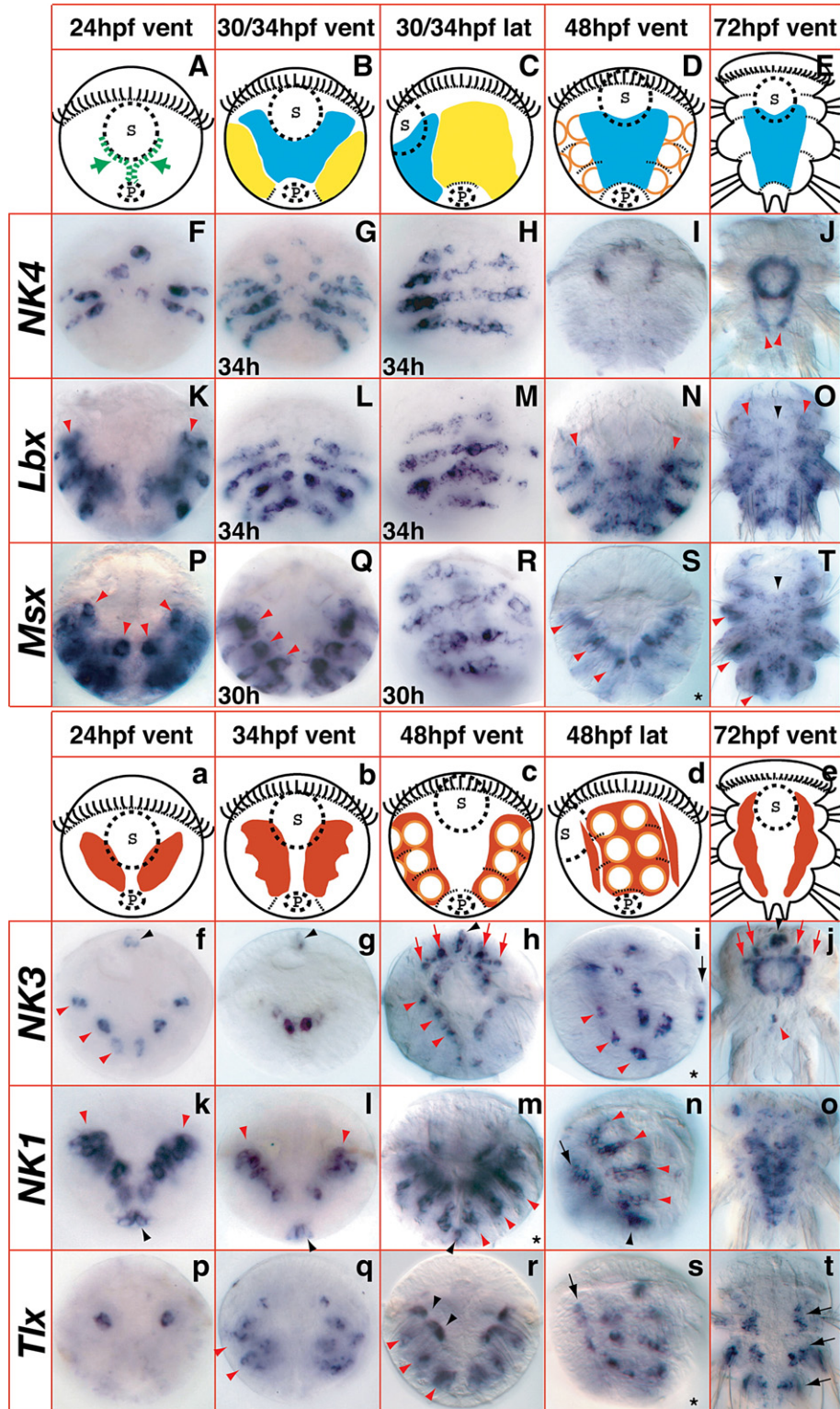
Fig. 3. The expression patterns of *NK* genes during trochophore development. 48- and 72-hpf larvae pictures are taken with Nomarski optics. Most of the images are ImageJ-flattened stacks except those indicated by an asterisk (see materials and methods). All the specimens are oriented anterior (or apical) up, vent: ventral view, lat: lateral view. All lateral views are oriented ventral side to the left. See text for the detailed descriptions of patterns. (A–E and a–e) Schematic descriptions of trochophore development and metamorphosis showing lateral epidermal ectoderm in yellow, neural ectoderm in blue, trunk mesoderm in red, the early ventral convergence of ectoderm in green and the setal sacs as orange circles; S: stomodeum, P: proctodaeum. For a given gene, the same type of arrow is used to indicate continuous expression in some tissues. (F–J) *Pdu–NK4*; red arrowheads: dorsal mesoderm. (K–O) *Pdu–Lbx*; red arrowheads: lateral mesoderm; black arrowhead: neuroectoderm. (P–T) *Pdu–Msx*; red arrowhead: lateral then parapodial mesoderm; black arrowhead: neuroectoderm. (f–j) *Pdu–NK3*; black arrowhead: unpaired brain cells, black arrow: dorsal nerve cells; red arrows: head mesoderm; red arrowheads: mesoderm. (k–o) *Pdu–NK1*; black arrowhead: proctodeal cells; black arrowhead: ventral neural cells; red arrowheads: parapodial muscle precursors. (p–t) *Pdu–Tlx*; black arrowheads: cells located deep in setal sacs probably chaetoblasts; red arrowheads: lateral mesodermal cells; black arrow: lateral cells in the neuroectoderm. See text for detailed description of more conspicuous aspects of each gene expression.
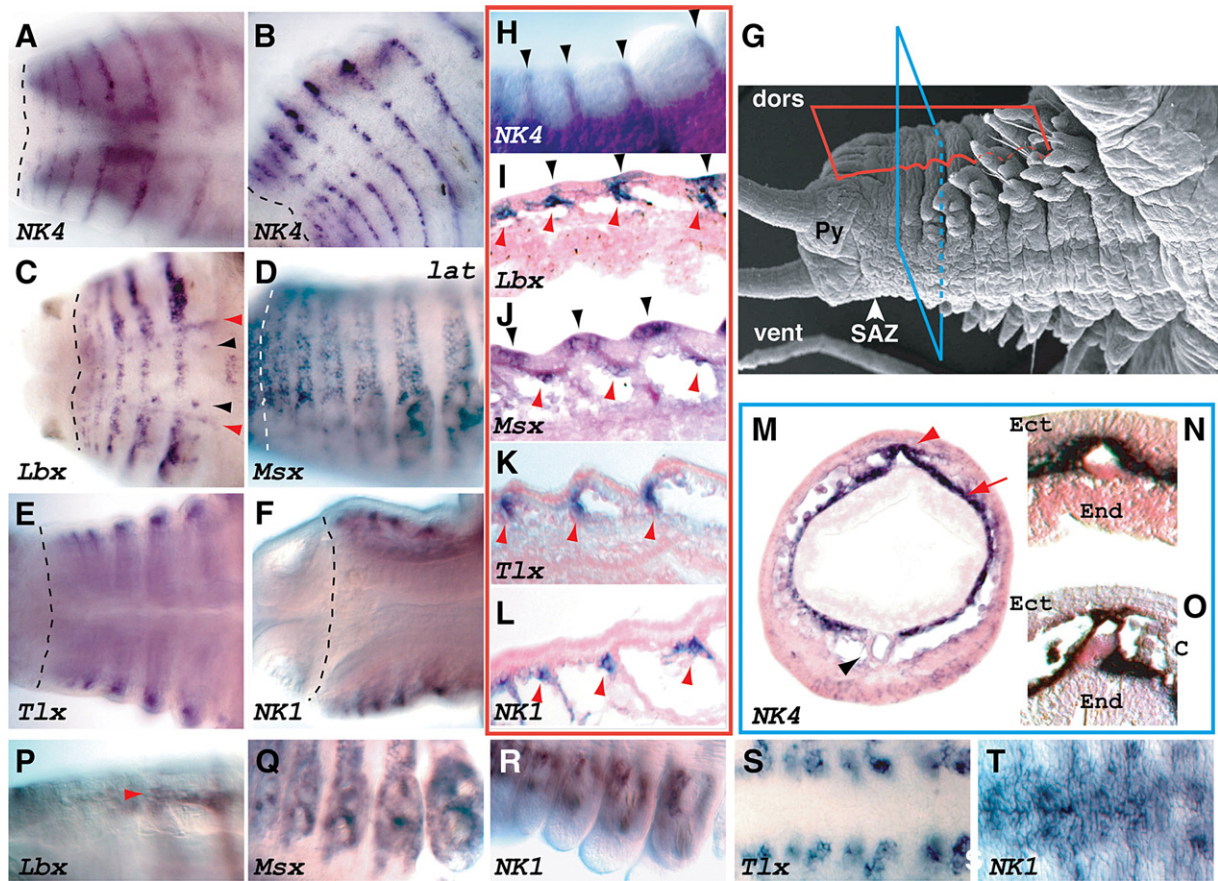
Fig. 4. The expression patterns of *NK* genes during juvenile posterior growth. All pictures were taken on regenerated posterior parts 8 days after amputation, showing 6–12 new segment anlagen. Posterior is on the left for all pictures except M, N, O. The dotted line is the anterior delimitation of the pygidium and immediately anterior to it lies the segment addition zone (SAZ) (de Rosa et al., 2005) (A–B) *Pdu–NK4*, ventral views with the stained gut (left) or with the gut removed (right). (C) *Pdu–Lbx*, ventral view. (D) *Pdu–Msx*, lateral view. (E) *Pdu–Tlx*, frontal optical section. (F) *Pdu–NK1*, frontal optical section. (G) Ventral–lateral view in scanning electron microscopy of a 8-day regenerated posterior part showing 7 segment anlagen in sequential differentiation; the red and blue squares indicate the approximate section plane locations for (H–O); Py: pygidium; SAZ: segment addition zone. (H–L) Lateral details of frontal tissue sections (*Pdu–Lbx, –Msx, –Tlx, –NK1*) or frontal optical section (*Pdu–NK4*) showing ectodermal stripes (black arrowheads) and mesodermal stripes (red arrowheads). (M–O) Transverse tissue sections showing for *Pdu–NK4*, the stained dorsal vessel (red arrowhead), the stained visceral mesoderm (red arrow) and the unstained ventral vessel (black arrowhead) and higher magnification views on the forming dorsal vessel in early (N) and more mature (O) segment anlagen. ect: ectoderm; end: gut endoderm; c: coelom. (P–R) Patterns in muscle precursors during posterior growth. Red arrowhead: dorsal longitudinal muscle precursors. (S–T) Patterns in the ventral neuroectoderm.

encircle the gut during posterior growth (Fig. 4A). A transverse section shows that the *NK4* probe stains a thin layer of cells covering the unstained gut endoderm (Fig. 4M, red arrow). Frontal sections show that the *NK4*-positive cells around the gut originate from mesodermal cells proliferating just anterior to the pygidium (not shown). On the dorsal side, *NK4* is expressed in the wall of the pulsatile dorsal vessel (Fig. 4M, red arrowhead) but not in the wall of the ventral vessel (Fig. 4M, black arrowhead). Transverse sections close to the pygidium show that the dorsal vessel wall is at first continuous with the visceral mesoderm (Fig. 4N) before the dorsal vessel progressively separates from the roof of the intestine in growing segments (Fig. 4O). During embryogenesis, there may be a similar mesodermal expression pattern. First, at 72 hpf, *NK4*-expressing cells form two dorsal longitudinal stripes in the three-segment larva (Fig. 3J, red arrowheads). To assess more accurately the location of these patterns, we used a Myosin Heavy Chain (*MHC*) probe in double WMISH. *MHC* appears to be expressed in all trunk muscles as well as the pharynx but not

in the visceral mesoderm or the pulsatile dorsal vessel. Double in situ with *MHC* (not shown) show that these stripes do not correspond to the dorsal longitudinal muscles but to a more dorsal part of the mesoderm. In 5-day-old larvae, the expression domain of *NK4* expands to cover the whole midgut region (Figure S1D), in the visceral mesoderm (although we cannot exclude an additional endodermal expression).

Msx, *Lbx*, *NK3*, *NK1* and *Tlx* are expressed from early stages in segmentally iterated cells in the mesodermal bands (Fig. 3, red arrowheads). The fate of these embryonic cells is not obvious but similar mesodermal expression domains during posterior growth for *Msx*, *Lbx* and *NK1* suggest that these cells are specific muscle precursors. *Lbx* is expressed in two sets of paired longitudinal stripes which are probably the precursors of the longitudinal muscles (Figs. 4C, P, red arrowheads; Figure S2C, white arrowheads) whereas *Msx* and *NK1* transcripts are found in proliferating cells located on the ventral–lateral sides of the trunk, which are incorporated in the forming parapodia and probably represent different subsets of parapodial muscle
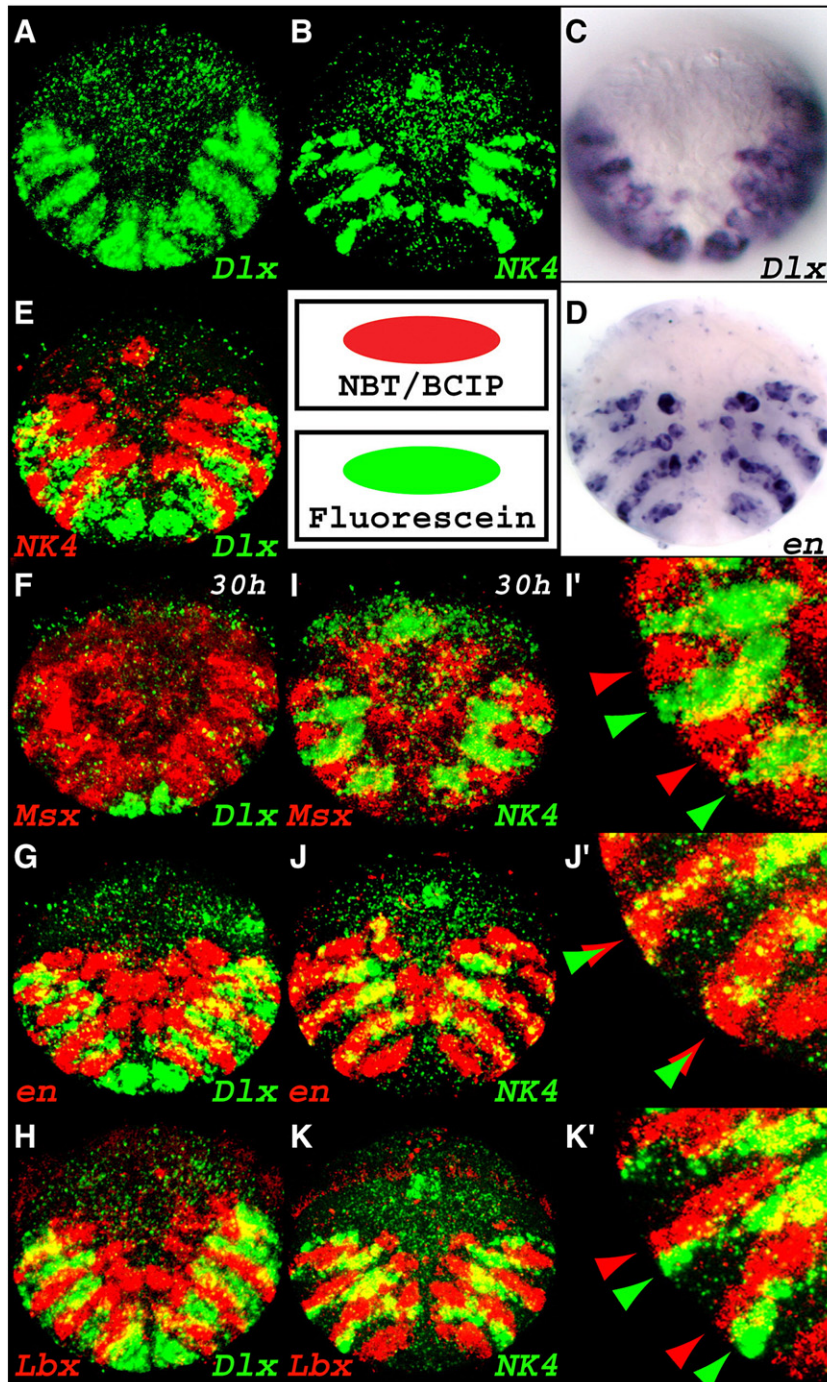
Fig. 5. Respective positions of *Pdu–NK4*, *Pdu–Lbx*, *Pdu–en*, *Pdu–Msx* and *Pdu–Dlx* ectodermal stripes in 34 hpf trochophores (30 hpf for *Msx* as indicated) as revealed with the double WMISH protocol (Jékely and Arendt, 2007). All pictures are ventral view projections of confocal 3D reconstructions. The interpretation of colour patterns is different from the classic fluorescent double WMISH. In this protocol, the NBT/BCIP tends to mask the fluorescent signal. The yellow pattern does not show co-expression but superimposed signals at different focal depth. Co-expression is shown by the partial or complete extinction of the fluorescent signal by the NBT/BCIP signal compared to the control with a fluorescent probe only. (A–B) Single probe fluorescent control patterns for *Pdu–Dlx* and *Pdu–NK4* (C–D) single probe NBT/BCIP control patterns for *Pdu–Dlx* and *Pdu–en* (E–K) double probe patterns, ventral views. The prototroch appears non-specifically stained by NBT/BCIP on some specimen. (I′–K′) High magnification views of (I–K) respectively, showing the overlap of *en*/*NK4* stripes and the non-overlap of *Msx*/*NK4* and *Lbx*/*NK4* stripes.

precursors (Figs. 4Q, R; Figure S2B, white arrowheads). As mentioned above, some *Tlx* cells during posterior growth contribute to the parapodia mesenchyme. The embryonic segmental pattern of *NK3* is more elusive as no obviously corresponding pattern is found during posterior growth. In the

hyposphere, *NK3* transcripts are at first present at 24 hpf in paired segmentally-iterated mesodermal cells (Fig. 3f, red arrowheads). This mesodermal expression undergoes complex dynamics. At 34 hpf, the segmental pairs disappear and are replaced by two deep groups of cells located anterior to the

proctodaeum (Fig. 3g, red arrowheads). At 48 hpf, paired mesodermal cells in deep location between the internal yolk-laden macromeres and the setal sacs are expressing *NK3* again (Figs. 3h, i, red arrowheads). At 72 hpf, we only see labelling of a small group of cells just anterior to the hindgut in the trunk of the small larva (Fig. 3j, red arrowhead) but no broad intestinal staining posterior to the pharynx that would suggest a role in the visceral mesoderm. During posterior growth, *NK3* shows a restricted pattern in some isolated cells of unknown origin dispersed in the gut endoderm (not shown).

Pdu–NK4 and Pdu–NK3 are expressed during the development of the pharynx

At 20 hpf, two large paired cells on the ventral surface just below the prototroch express *NK4* (Figure S1A). At 24 hpf, *NK4* is expressed in three small groups of internal cells around the forming stomodeum (Fig. 3F). These cells are positioned asymmetrically. This asymmetric expression undergoes complex dynamics and persists until at least 28 hpf (not shown). Then at 34 hpf, a new set of *NK4*-positive cells appear external but in contact with the stomodeum (Fig. 3G). These cells are located more internally near the dorsal (i.e. ultimately the posterior-most after reorientation) end of the stomodeum and are comprised of five small groups of cells: one anterior spot and two pairs of lateral spots. This staining evolves into an incomplete ring of cells in 48 hpf larvae (Fig. 3I) and a complete ring in 72 hpf (Fig. 3J) when the stomodeum shifts gradually to an anterior–posterior orientation and starts to transform into a functional pharynx. Meanwhile, *NK3*-expressing cells also participate in the formation of the pharynx. At 48 hpf, two bilateral small groups of cells start to surround the stomodeum (Fig. 3h) and at 72 hpf, the peri-stomodeal staining extends to form a tube. Apical views of 72 hpf larvae (Figures S1B,C) show that *NK4* and *NK3* are expressed in similar patterns in the posterior (still dorsal at this stage) part of the pre-pharynx (with *NK3* more anteriorly extended than *NK4*) in an ectomesodermal sheath (i.e. anterior mesoderm not produced by the 4d cell, Ackermann et al., 2005) surrounding the stomodeum *stricto sensu*. The fate of these cells is not known as most of the complicated musculature of the adult proboscis and pharynx derives from posterior mesoderm. Some other *NK3* expressing cells in the episphere (Figs. 3h, j, red arrows) probably represent ectomesoderm-derived head muscles (Ackermann et al., 2005).

Pdu–NK3, Pdu–Msx, Pdu–Lbx, Pdu–Tlx, Pdu–NK1 and Pdu–NK5 are expressed in the forming central and peripheral nervous systems

In the trochophore, the ventral neural ectoderm starts to thicken at 30 hpf and the VNC starts to differentiate before 48 hpf (Denes et al., 2007). The neural fields in the episphere that will form most of the brain follow the same path. *NK5* and *NK3* are the only genes expressed in the episphere, *NK5* broadly in the dorsal part of the future brain (Figure S1F) and *NK3* in small unpaired sets of cells (Figs. 3f–j, black arrow, black arrowhead). On the ventral part of the hyposphere of the

embryo as well as during posterior growth, two kinds of neural cells differentiate: the neurons of the VNC (central nervous system) and more lateral neurons associated with the parapodia (peripheral nervous system). *NK1* is expressed in the future VNC. Expression starts at 36 hpf in a few ventral cells (not shown) then spreads rapidly to a large number of cells in the future VNC as well as the peristomial ectoderm at 48 hpf (Figure S1E) and 72 hpf (Fig. 3o). The same expression is seen during posterior growth (Fig. 4T). *Lbx* and *Msx* are expressed in a restricted number of cells in each neural segment during trochophore development (Fig. 3O, black arrowhead and not shown) and posterior growth (Fig. 4C, black arrowheads and not shown). *Tlx* and *NK5* are expressed in very lateral domains of the ventral neural plate (Fig. 3s, black arrow, Fig. 4S, Figure S1F) as well as in group of cells at the basis of the parapodia. Comparison with the pattern obtained with an anti-acetylated tubulin antibody (not shown) suggests that these more lateral aspects are also differentiating nerve cells belonging to the peripheral nervous system. *Msx* is also persistently expressed in cells of the parapodia anlagen that could be sensory neurons (Fig. 4Q).

Discussion

The *NK* genes of *Platynereis* seem to be involved in a diversity of functions in the epidermal and neural ectoderm, mesoderm and digestive tract. From comparisons with insects and vertebrates, we can infer for each gene its likely ancestral functions. These comparisons suggest that *Urbilateria* was already using its *NK* cluster genes in coordinated ways in the patterning of multiple structures and organs.

The urbilaterian NK cluster functioned as a mesodermal patterning cluster

All *NK* genes tested except *NK5/Hmx* are expressed in the mesoderm of *Platynereis*. Notably, *Pdu–Msx*, *Pdu–Lbx*, *Pdu–NK1* are expressed in presumptive myoblasts that will give rise to different segmental muscles. The non-overlapping patterns of the three genes in the longitudinal muscles and parapodial muscles suggest that they may provide identity information required for the differentiation of these different muscles. This situation is highly reminiscent of what is seen in the fly (*msh*: Jagla et al., 1999, Nose et al., 1998; *NK1/S59/Slouch*: Knirr et al., 1999; *ladybird*: Jagla et al., 1998; *C15/Clawless*: Jagla et al., 2001) in which the *NK* genes are described as identity genes that specify the different muscle precursor cells set apart early in development. In vertebrates, *Msx* and *Lbx* genes are expressed in somatic muscle precursors: *Msx1* is expressed transiently in somite cells that will give rise to dorsal dermis (Houzelstein et al., 2000), limb muscles and intercostal muscles (Houzelstein et al., 1999; Bendall et al., 1999); *Lbx* genes (mouse *Lbx1*: Gross et al., 2000; Brohmann et al., 2000; chick *Lbx1*: Dietrich et al., 1998; chick *Lbx3*: Kanamoto et al., 2006) are expressed in hypaxial somite cells that will migrate to form neck and limb muscles. These data make a function in the developing somatic musculature of *Urbilateria* very likely for *Msx* and *Lbx* at least.

NK4 is expressed in the gut in 5-day-old larvae, roughly at the time when a functional digestive system forms in *Platynereis*. The staining includes the mesoderm-derived part of the gut. During posterior growth, *NK4* is expressed in the visceral part of the proliferating and differentiating mesoderm from which both the visceral musculature and the walls of the dorsal vessel originate. In the fly, *tinman* mutants lack a functional gut (Azpiazu and Frasch, 1993). In vertebrates, some *NK4* orthologues are expressed in more or less axially extended regions of the mesoderm-derived tissues of the developing digestive system (mouse *Nkx2.3*: Pabst et al., 1997; mouse *Nkx2.5*: Lints et al., 1993; chick *Nkx2.3* and *Nkx2.5*: Buchberger et al., 1996). The absence of a function of *Pdu–NK3* in the patterning of the trunk mesoderm is surprising. The involvement of this gene in the fly visceral mesoderm (Azpiazu and Frasch, 1993) and in vertebrate gut mesoderm (mouse *Nkx3.1*: Tanaka et al., 1999; mouse *Nkx3.2*: Tribioli et al., 1997; chick *Nkx3.2*: Schneider et al., 1999; frog *Nkx3.2*: Newman et al., 1997; frog *Nkx3.3*: Newman and Krieg, 1999) suggests that this is indeed an ancestral function, lost in *Platynereis*.

In summary, *NK* genes probably played roles in the differentiation of both the somatic (*Msx* and *Lbx* at least) and the visceral (*NK4* and *NK3*) musculature of *Urbilateria*. This implies that these two muscle sets were already distinct as would be expected if *Urbilateria* was a coelomate animal.

The expression of NK4 in an annelid confirms that the vertebrate heart derives from an urbilaterian dorsal pulsatile vessel

NK4 expression in *Platynereis* confirms the linkage that exists between the pulsatile part of the circulatory system or "heart" and the visceral mesoderm. The ontogenetic connection appears clearly in *Platynereis* as the dorsal vessel initially emerges from the roof of the gut during posterior growth. The visceral mesoderm of *Platynereis* gives a circular musculature responsible for the gut peristaltic contractions but also it forms a grid-like blood sinus around the gut endoderm. *Platynereis* blood vessels, including the dorsal vessel, are simple tubes formed in intercoelomic spaces and covered by a single layer of myo-epithelial cells without endothelium (Nakao, 1974 on *Nereis japonica*, and our observations on *Platynereis*). It has to be noticed that the dorsal vessel is not the only vessel that is capable of contractile activity. The segmental lateral vessels emerging from the gut sinus propel the blood towards the parapodia through their own independent contractile activity easily seen on chemically relaxed animals (our observations). *NK4* is not expressed in contractile lateral vessels, showing that *NK4* is not just a cell differentiation gene linked to the presence of autonomously contractile myo-epithelial cells but that it has indeed a true patterning function, as in the fly and vertebrates.

In flies, *tin* mutants lack a dorsal vessel (Azpiazu and Frasch, 1993). In vertebrates, most *tin* orthologues are expressed either very early in the paired ventral primordia that will fuse to give rise to the heart tube (mouse *Nkx2.5*: Lints et al., 1993; chick *Nkx2.5*: Buchberger et al., 1996; frog *Nkx2.3*: Evans et al., 1995; frog *Nkx2.5*: Tonissen et al., 1994; frog *Nkx2.10*:

Newman et al., 2000; teleost *Nkx2.5* and *Nkx2.7*: Lee et al., 1996) and/or more lately in the differentiating heart (mouse *Nkx2.6*: Biben et al., 1998; chick *Nkx2.3*: Buchberger et al., 1996; chick *Nkx2.8*: Brand et al., 1997). The *Platynereis* data confirm the hypothesis of a phylogenetic continuity between the simple protostome dorsal vessel and the complex vertebrate heart. The restriction of *Pdu–NK4* expression to the dorsal part of the circulatory system brings a crucial new argument in favour of the dorsal–ventral axis inversion in the ancestor of chordates (Arendt and Nubler-Jung, 1994). *tin* expression in *Drosophila* is also restricted to the dorsal side but the blood vessel system in insects is always reduced to the dorsal vessel. The closed metameric circulatory system of *Platynereis* may be close to the ancestral state that existed in *Urbilateria*, compared to the more derived situations in arthropods (fusion of the blood and coelom compartments into a mixocoel) and vertebrates (appearance of a complex heart, reviewed in Simoes-Costa et al., 2005). In a chordate ancestor, the dorsal vessel was brought in ventral position as the dorsal–ventral axis was inverted and its anterior part gradually evolved into a heart by folding on itself. Other striking evidence for this scenario come from the chordate *Branchiostoma* (Holland et al., 2003; Luke et al., 2004) which has no true heart but in which *NK4* is expressed in a vessel forming ventrally to the gut.

Ancestral bilaterian NK genes participated in the anterior digestive tract formation

Pdu–NK3 and *Pdu–NK4* are likely involved in stomodeal and pharyngeal development in *Platynereis*. Bilaterian-wide comparisons make it clear that *NK4* must have played a role in patterning the foregut/pharynx region of *Urbilateria*. In the fruitfly, around gastrulation time, *tin* (but not *NK3/bagpipe*) is expressed in an anterior region of the embryo that represents the primordia of the oesophagus and epipharynx (de Velasco et al., 2006). In vertebrates, *NK4/tinman* orthologues are variously expressed in the oral ectoderm, the pharyngeal endoderm and the branchial arches endoderm and ectoderm (mouse *Nkx2.3*: Biben et al., 2002; mouse *Nkx2.5*: Lints et al., 1993; mouse *Nkx2.6*: Nikolova et al., 1997, Biben et al., 1998; chick *Nkx2.3* and *Nkx2.5*: Buchberger et al., 1996; chick *Nkx2.8*: Brand et al., 1997; frog *Nkx2.3*: Evans et al., 1995; frog *Nkx2.5*: Tonissen et al., 1994; frog *Nkx2.10*: Newman et al., 2000; teleost *Nkx2.3* and *Nkx2.7*: Lee et al., 1996). Compound mouse mutants for *Nkx2.3/Nkx2.5* show a grossly abnormal pharyngeal development with no trace of a pharynx endoderm (Tanaka et al., 2001). Vertebrates *NK3* orthologues are also expressed in the pharyngeal endoderm and arches and some of their derivatives such as the tongue and teeth (mouse *Nkx3.1*: Tanaka et al., 1999; mouse *Nkx3.2*: Tribioli et al., 1997; chick *Nkx3.2*: Schneider et al., 1999; frog *Nkx3.3*: Newman and Krieg, 1999). Another hint in favour of an ancestral role of a part of the *NK* cluster in stomodeal/pharyngeal development is the function of *NK2.1*, another NKL gene. *NK2.1* has not been found in any bilaterian *NK* cluster to date but its primary structure is very close to *NK4* and *NK3*, which suggests that it appeared in the same wave of gene duplication that produced the *NK* cluster in a

metazoan ancestor. In the fruitfly, *NK2.1/Scarecrow* is expressed in the developing pharynx (Zaffran et al., 2000). In vertebrates, *Nkx2.1* is involved in thyroid and lung development, two foregut derivatives (Minoo et al., 1999) and in *Platynereis*, it is expressed in the developing stomodeal bulb (Tessmar-Raible et al., 2007).

Patterning of neural precursor fates in the central and peripheral nervous system

Denes et al. (2007) have shown the extensive genetic similarities in the early medial–lateral patterning between the VNC of *Platynereis* and the neural tube of vertebrates. This work strongly supports the existence of a condensed nervous system in *Urbilateria* thus contradicting earlier interpretations based on hemichordate data (Lowe et al., 2003; Lowe et al., 2006). The similar overlapping expression of early patterning genes suggests that homologies exist between the medio-lateral parts (often referred as "columns") of annelid and vertebrate nervous system. From a *sim+* midline to lateral, Denes and coauthors divide both nervous systems in five paired columns: a *Nk2.2/Nk6* column from which serotoninergic motoneurons emerge, a *Nk6/pax6* column giving birth to cholinergic motoneurons, a *pax6/pax3/7* column and a *pax3/7/ Msx* column producing neurons with interneuronal molecular signatures and a *Msx/Dlx* lateral domain harboring sensory neurons. The embryonic pattern of the gene *Pdu–Msx* has already been described in some details in Denes et al. (2007) and spans the neural/non-neural border in both vertebrates and *Platynereis*. The expression of *Pdu–Msx* during posterior growth in *Platynereis* described in this work suggests a correspondence between the dorsal part of the vertebrate neural tube and ventral–lateral ectodermal domains near parapodia in *Platynereis* (thus located outside of the VNC ganglia). With this correspondence in mind, we can try to compare the other *NK* genes patterns. The timing of *NK1*, *NK5*, *Lbx* and *Tlx* expressions in presumed neural precursors in *Platynereis* suggest that they are involved in late aspects of neuronal differentiation. The wide distribution of neural cells labelled by *Pdu–NK1* seen in the forming VNC of *Platynereis* corresponds well with the expression of *NK1* orthologues in the ventral neural tube of vertebrates (Schubert et al., 1995; Simon and Lufkin, 2003; Bae et al., 2004) and the neuronal pattern of *NK1*/S59 in the fly VNC (Jagla et al., 2001). Conversely, the vertebrate *Tlx* orthologues (mouse *Tlx1* and *Tlx3*: Raju et al., 1993, Qian et al., 2002, chick *Tlx1* and *Tlx3*: Logan et al., 1998) are expressed in the dorsal neural tube, dorsal root sensory neurons and cranial sensory ganglia, corresponding well with their expression in peripheral neurons in *Platynereis*. *Lbx* and *Hmx* expression fit less well with the Denes et al. prototype. The peripheral-only *NK5* expression in *Platynereis* corresponds partially to the central and peripheral expression of vertebrates *Hmx* genes (Wang et al., 2000) and the restricted distribution of *Pdu–Lbx* in VNC ganglia corresponds rather to the role of *ladybird* in the fly VNC (De Graeve et al., 2004) than to the wider patterns of *Lbx1* genes in the dorsal vertebrate neural tube (Kruger et al., 2002;

Schubert et al., 2001). All these similarities make *Msx*, *Lbx*, *Tlx*, *NK1* and *NK5* likely players in the development of the condensed nervous system present in the ancestral bilaterian and the medio-lateral locations of the neural cells expressing these genes correspond well with the ancestral regionalization of the nervous system proposed by Denes and coauthors for three of the *NK* genes (*Msx*, *NK1* and *Tlx*).

Role of the NK cluster in segment formation: annelid innovation or Urbilaterian legacy?

Five *NK* genes are involved in segment-polarity like patterns in the ectoderm (*Pdu–NK4*, *Pdu–Lbx* and *Pdu–Msx*) and mesoderm (*Pdu–Msx*, *Pdu–Lbx*, *Pdu–Tlx* and *Pdu–NK1*) of *Platynereis*. These striped patterns of *NK* genes appear before segments are morphologically visible and persist during posterior growth in differentiating segment anlagen as ectoderm-derived organs (nervous system, parapodia) and mesoderm-derived organs (blood vessels, muscle fibres and nephridia) appear. This strongly suggests that the *NK* gene expression carries positional patterning information as segment anlagen form and grow through cell divisions. In the ectoderm, *Pdu–NK4* and *Pdu–Lbx* may function as segment polarity genes on each side of the segmental boundary and *Pdu–Msx* may specify mid-segment ectoderm. The mesodermal stripes of *Pdu–Msx*, *Pdu–Lbx*, *Pdu–Tlx* and *Pdu–NK1* are associated with the segmented mesodermal epithelia that surround the coelomic cavities or somites (Bartolomaeus, 1994). *Platynereis* shows no recognisable somite structure during the embryonic development of the first three segments of the larva as internal organs are produced directly from the early mesodermal bands. This probably explains why no clear mesodermal *NK* stripes appear in this first phase of development. Remarkably, the patterns of the five genes, although there are some overlapping areas and "empty spaces", are complementary, covering most of the ectodermal and mesodermal segments (Fig. 6). This suggests that *NK* genes might be working in a concerted way to pattern individual segments.

What does the likely involvement of *NK* genes in segmental patterning in an annelid tell us about the origin and evolution of segmentation? Our work is the first description of complementary segment-polarity patterns for *NK* genes in a bilaterian. The only other *NK* expression patterns described in annelids are those of the *Lox10* gene (probably a *NK2.1* orthologue; Nardelli-Haefliger and Shankland, 1993) and *Msx* (Master et al., 1996) in the leech, and neither of these shows a segment-polarity-like pattern. In *Drosophila*, most of the *NK* genes do not regulate segmentation. However, one particular gene, orthologous to *Pdu–Lbx* and the vertebrate *Lbx* genes, *ladybird*, has a segment-polarity function (Jagla et al., 1997). Almost none of the *NK* genes show a segment-polarity-like function in vertebrates, neither during somitogenesis nor during hindbrain segmentation. The only exception is the murine gene *Msx1* that shows a striped pattern in the dorsal somite-derived mesenchyme (Houzelstein et al., 2000) but such a pattern has not been described in other vertebrates. These comparisons of *NK* expression and functions where they are available in
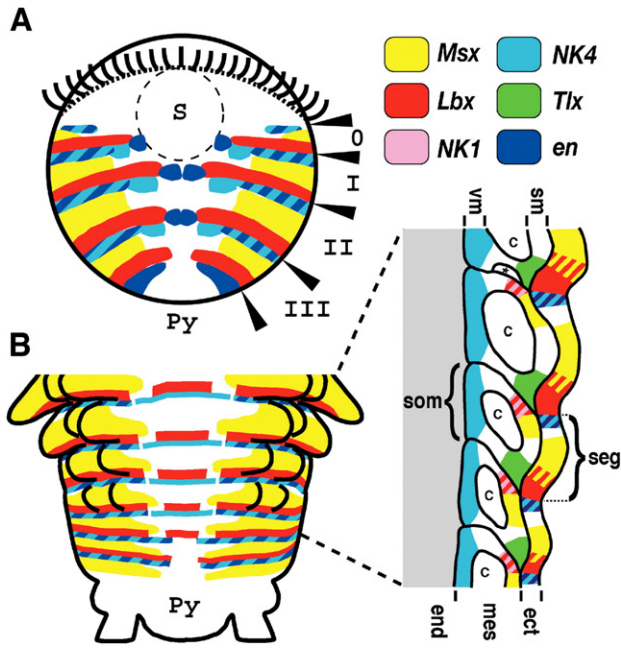
Fig. 6. Interpretative schemes of the respective location of *NK* gene and *engrailed* patterns in ectodermal and mesodermal segments in a 34 hpf trochophore, in ventral view (A) and during posterior growth, in ventral external view and in frontal section (B). Anterior (apical) is up for both schemes. Overlapping domains of expression in panel B are only approximate, as no double WMISH has been obtained yet on regenerates. At 34 hpf, *Pdu–Msx* has started to fade in the ectoderm at this stage but its 30 hpf expression corresponds roughly to the *Pdu–Dlx* stripes. In posterior growth, notice that mesodermal segments are shifted anteriorly respective to external segmentation and are in register with neural segmentation (VNC ganglia); S: stomodeum, Py: pygidium, 0: transient non-setigerous first segment anlagen that later fuses with the head, I–III: setigerous segment anlagen, ect: ectoderm, mes: mesoderm, sm: somatic mesoderm, vm: visceral mesoderm, end: endoderm, c: coelomic cavity, seg: externally visible segment, som: somite or mesodermal segment, asterisk: lateral blood vessel.

bilaterians plead at first glance in favour of the interpretation that the segment polarity patterns (and presumed functions) evolved specifically in annelids. However, when taking into account all available data in the ancestral segmentation debate, a different scenario seems possible. In the fly, initiation of *ladybird* expression is coincident with and dependent on *wingless*, then later the relation is reversed with *ladybird* governing the maintenance of *wingless* in dorsal epidermis. What we see in *Platynereis* is similar: *Pdu–Lbx* and *Pdu–Wnt1* are expressed in overlapping domains just anterior to *Pdu–en* stripes. It seems unlikely that the three genes *engrailed*, *wingless* and *ladybird* might have been recruited independently to fulfil segment formation functions in the same spatial relationship in arthropods and annelids. The *Pdu–Lbx* pattern thus reinforces our published hypothesis that the last common ancestor of protostomes was segmented and that annelid segments are homologous with arthropod parasegments (Prud'homme et al., 2003). Combined with the involvement of the Notch/delta pathway in the formation of the segments of a spider (Stollewerk et al., 2003; Schoppmeier and Damen, 2005) in a way comparable to what is seen in vertebrate somitogenesis, these data, in our opinion, are now supporting strongly a metameric bilaterian ancestor. If we postulate this annelid-like

metameric ancestor, it seems unlikely to us that the ancient, related and initially clustered *NK* genes might have acquired such coordinated expression domains in ectoderm and mesoderm late in evolutionary history, only during annelid evolution. Thus, the situation in *Platynereis* may reflect closely the role of *NK* genes in the segmentation of *Urbilateria*. As a corollary, the segmentation function should have been almost completely lost in the lineage leading to the fruitfly and entirely in the lineage leading to vertebrates. It is noticeable in this respect that *NK4* and *en* have overlapping striped expression patterns in *Platynereis*. If this reflects the ancestral urbilaterian condition, the two homeobox genes might have become functionally redundant in defining segment borders. Eventually, *en* might have won the competition to become a key gene in shaping these borders in arthropod ancestors (Larsen et al., 2003), thus evicting *NK4* ectodermal expression. In annelids, the fact that *en* is not found involved in segment patterning in several species, including the leech *Helobdella* (Seaver and Shankland, 2001), *Chaetopterus* (Seaver et al., 2001), *Hydroides* and *Capitella* (Seaver and Kaneshige, 2006) might be due to the reciprocal functional eviction by *NK4*. An obvious direction for testing those speculations in the future would be to study *NK* gene expression patterns in a panel of arthropods (including a chelicerate, a myriapod and a crustacean), in the accessible annelid species mentioned above, in onychophorans (which are related to arthropods, but show a number of annelid-like characters, such as mesodermal somites) and possibly also in a segmented mollusc (such as chitons). Imagining why the *NK* function in segmentation would have been lost in vertebrates is also quite challenging. The striped ectodermal expression has probably been lost quite simply because vertebrates are not segmented externally. On the mesoderm side, it is tempting in the context of the segmented *Urbilateria* hypothesis to propose that annelid somites might be homologous with vertebrate somites but the absence of striped *NK* expression in vertebrate somites would rather suggest that they are not related in a simple way. Indeed, the annelid somites are essentially delimitating the coelom, whereas the vertebrate somites are only the dorsal part of the mesoderm and do not contribute to the coelom formation (which is produced by the ventral unsegmented lateral plate mesoderm).

Conclusion

The complexity and correlated aspects of ancestral *NK* gene functions suggested by bilaterian comparisons are altogether supporting well the idea of a complex coelomate *Urbilateria* with a blood circulatory system and a condensed central nervous system. The involvement of five *NK* genes (*Msx*, *NK4*, *Lbx*, *Tlx*, *NK1*) in annelid segment formation is a striking discovery, the evolutionary origin of which obviously deserves investigation. The genetic similarities at the level of segment polarity genes already discovered between *Platynereis* and arthropods (*engrailed*, *wingless*, *ladybird*) strongly support a metameric protostome ancestor because these similarities establish spatial correspondence within individual segments in both groups. In this respect, the absence of similarities between

Platynereis and vertebrates in *NK* segmental expression in the mesoderm is noticeable. Discovering similarities in segment patterning between protostomes and deuterostomes thus remains a crucial issue in the segmentation debate.

Materials and methods

Breeding culture, embryo collection and regenerating animals

Animals were obtained from a breeding culture established in Gif-sur-Yvette according to the protocol of Fischer and Dorresteijn (www.platynereis.de). Trochophores and larvae were collected and fixed as previously described (Tessmar-Raible et al., 2005). Regenerated posterior parts were obtained as previously described (de Rosa et al., 2005) except fixation was performed in PBS+0.1% Tween20+4% paraformaldehyde.

Cloning of cDNAs, phylogenetic analyses

Short fragments for *Pdu–NK1*, *–NK3*, *–NK4* and *–Tlx* were obtained with one-side or two-side specific PCR on 24 hpf and 48 hpf cDNA libraries with homeobox degenerate primers either using conventional degenerate PCR protocols (*Tlx* specific primers: forward GGNYTNCCNTAYCARAAYMG-NACNCC, forward nested CCNTAYCARAAYMGNACNCCNCC, reverse TTYTGRAACCANGTYTTNACYTG, reverse nested AACCANGTYTTNA-CYTGNGCRTC) or a 3′ specific protocol combined with primers in the library vector (generic homeobox reverse primers: WFQNRR CGGGATC-CCKNCKRTTYTGRAACCA and nested KIWFQN GGAATTCRTTYT-GRAACCANAYYTT). Larger cDNA fragments were amplified on 24 hpf and 48 hpf cDNA libraries with the SMART(tm) PCR protocol (Clontech). A *Msx* fragment was amplified using sequence information provided by the Arendt group (EMBL, Heidelberg). A *Lbx* cDNA was identified in a EST collection (Raible et al., 2005).

Neighbor-Joining gene trees were made with PAUP 4.0. Accession numbers: Honeybee, *vnd*, XP001121493; *Scro*, XP394578; *tin*, XP001120208; *bap*, XP001120208; *lb*, XP001120087; *C15*, XP001119904; *Slou*, XP001121341; *DropA*, XP001120268; *DropB*, XP001120318; *dll*, XP001122433. Fruitfly, *vnd*, NP001036253; *Scro*, NP001015473; *tin*, NP524433; *bap*, NP732637; *lbl*, NP524434; *lbe*, NP524435; *C15*, NP476873; *H6*, NP524951; *Slou*, NP476657; *Drop*, NP477324; *dll*, NP726486. Mouse, *TTF1*, NP033411; *Nkx2.4*, NP075993; *Nkx2.2*, NP035049; *Nkx2.3*, NP035049; *Nkx2.5*, NP032726, *Nkx2.3*, NP032725; *Nkx2.6*, NP035050; *Nkx2.8*, XP999232; *Nkx3.1*, NP035051; *Nkx3.2*, NP031550; *Lbx1*, NP034821; *Lbx2*, NP034822; *Tlx1*, NP068701; *Tlx2*, NP033418; *Tlx3*, NP064300; *Hmx1*, NP034575; *Hmx2*, NP666110; *Hmx3*, NP032283; *Nkx1.1*, XP144267; *Nkx1.2*, NP033149; *Msx1*, NP034965; *Msx2*, NP032610; *Msx3*, NP034966; *dlx1*, NP034183; *dlx2*, NP034184; *dlx3*, NP034185; *dlx4*, NP031893; *dlx5*, NP034186. *Platynereis*, *Nk2.1*, CAJ38809; *Nk2.2*, unpublished sequence courtesy of Detlev Arendt, *NK4*, ABQ10640; *NK3*, ABQ10641; *Lbx*, ABQ10642; *Tlx*, ABQ10643; *NK5*, ABQ10644; *NK1*, CAJ38797; *Msx*, CAJ38810; *Dlx*, CAJ38799.

Whole mount in situ hybridizations (WMISH)

Single and double-probe WMISH were carried out using published protocols (Tessmar-Raible et al., 2005; Jékely and Arendt, 2007). For regenerated posterior parts, two proteinase K treatments were used in parallel (100 μg/ml for 3 min or 10 μg/ml for 10 min) to optimize labelling in the mesoderm and ectoderm respectively. 5–10 μm tissue sections in paraffin on WMISH were made according to an usual protocol (Prud'homme et al., 2003).

Light and confocal microscopy, image processing

Stained embryo picture Z-stacks were taken manually on a light microscope and Zprojection images were made with ImageJ 1.36b. Confocal picture Z-stacks were taken on a Leica Sp2 confocal microscope and images were 3D reconstructed with Metamorph.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.013.

References

Ackermann, C., Dorresteijn, A., Fischer, A., 2005. Clonal domains in postlarval *Platynereis dumerilii* (Annelida: Polychaeta). J. Morphol. 266, 258–280.

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B., de Rosa, R., 2000. The new animal phylogeny: reliability and implications. Proc. Natl. Acad. Sci. U. S. A. 97, 4453–4456.

Arendt, D., Nubler-Jung, K., 1994. Inversion of dorsoventral axis? Nature 371, 26.

Azpiazu, N., Frasch, M., 1993. tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. Genes Dev. 7, 1325–1340.

Bae, Y.K., Shimizu, T., Muraoka, O., Yabe, T., Hirata, T., Nojima, H., Hirano, T., Hibi, M., 2004. Expression of sax1/nkx1.2 and sax2/nkx1.1 in zebrafish. Gene Expr. Patterns 4, 481–486.

Balavoine, G., Adoutte, A., 2003. The segmented Urbilateria: a testable scenario. Integr. Comp. Biol. 43, 137–147.

Bartolomaeus, T., 1994. On the ultrastructure of the coelomic lining in the Annelida, Sipuncula and Echiura. In: Ax, P. (Ed.), Microfauna Marina, vol. 9. Gustav Fischer Verlag, Stuttgart, pp. 171–220.

Bendall, A.J., Ding, J., Hu, G., Shen, M.M., Abate-Shen, C., 1999. Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. Development 126, 4965–4976.

Biben, C., Hatzistavrou, T., Harvey, R.P., 1998. Expression of NK-2 class homeobox gene Nkx2–6 in foregut endoderm and heart. Mech. Dev. 73, 125–127.

Biben, C., Wang, C.C., Harvey, R.P., 2002. NK-2 class homeobox genes and pharyngeal/oral patterning: Nkx2–3 is required for salivary gland and tooth morphogenesis. Int. J. Dev. Biol. 46, 415–422.

Bodmer, R., 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. Development 118, 719–729.

Brand, T., Andree, B., Schneider, A., Buchberger, A., Arnold, H.H., 1997. Chicken NKx2–8, a novel homeobox gene expressed during early heart and foregut development. Mech. Dev. 64, 53–59.

Brohmann, H., Jagla, K., Birchmeier, C., 2000. The role of Lbx1 in migration of muscle precursor cells. Development 127, 437–445.

Buchberger, A., Pabst, O., Brand, T., Seidl, K., Arnold, H.H., 1996. Chick NKx-2.3 represents a novel family member of vertebrate homologues to the Drosophila homeobox gene tinman: differential expression of cNKx-2.3 and cNKx-2.5 during heart and gut development. Mech. Dev. 56, 151–163.

Damen, W.G., 2007. Evolutionary conservation and divergence of the segmentation process in arthropods. Dev. Dyn. 236, 1379–1391.

Davis, G.K., Patel, N.H., 1999. The origin and evolution of segmentation. Trends Cell Biol. 9, M68–M72.

De Graeve, F., Jagla, T., Daponte, J.P., Rickert, C., Dastugue, B., Urban, J., Jagla, K., 2004. The ladybird homeobox genes are essential for

the specification of a subpopulation of neural cells. Dev. Biol. 270, 122–134.

Denes, A.S., Jekely, G., Steinmetz, P.R., Raible, F., Snyman, H., Prud'homme, B., Ferrier, D.E., Balavoine, G., Arendt, D., 2007. Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. Cell 129, 277–288.

de Rosa, R., Prud'homme, B., Balavoine, G., 2005. Caudal and even-skipped in the annelid *Platynereis dumerilii* and the ancestry of posterior growth. Evol. Dev. 7, 574–587.

de Velasco, B., Mandal, L., Mkrtchyan, M., Hartenstein, V., 2006. Subdivision and developmental fate of the head mesoderm in *Drosophila melanogaster*. Dev. Genes Evol. 216, 39–51.

Dietrich, S., Schubert, F.R., Healy, C., Sharpe, P.T., Lumsden, A., 1998. Specification of the hypaxial musculature. Development 125, 2235–2249.

Dorresteijn, A.W.C., O'Grady, B., Fischer, A., Porchet-Henneré, E., Boilly-Marer, Y., 1993. Molecular specification of cell lines in the embryo of Platynereis (Annelida). Roux's Arch. Dev. Biol. 202, 260–269.

Evans, S.M., Yan, W., Murillo, M.P., Ponce, J., Papalopulu, N., 1995. tinman, a Drosophila homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: XNkx-2.3, a second vertebrate homologue of tinman. Development 121, 3889–3899.

Gross, M.K., Moran-Rivard, L., Velasquez, T., Nakatsu, M.N., Jagla, K., Goulding, M., 2000. Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. Development 127, 413–424.

Hartenstein, V., Mandal, L., 2006. The blood/vascular system in a phylogenetic perspective. BioEssays 28, 1203–1210.

Harvey, R.P., 1996. NK-2 homeobox genes and heart development. Dev. Biol. 178, 203–216.

Holland, N.D., Venkatesh, T.V., Holland, L.Z., Jacobs, D.K., Bodmer, R., 2003. AmphiNk2-tin, an amphioxus homeobox gene expressed in myocardial progenitors: insights into evolution of the vertebrate heart. Dev. Biol. 255, 128–137.

Houzelstein, D., Auda-Boucher, G., Cheraud, Y., Rouaud, T., Blanc, I., Tajbakhsh, S., Buckingham, M.E., Fontaine-Perus, J., Robert, B., 1999. The homeobox gene Msx1 is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb. Development 126, 2689–2701.

Houzelstein, D., Cheraud, Y., Auda-Boucher, G., Fontaine-Perus, J., Robert, B., 2000. The expression of the homeobox gene Msx1 reveals two populations of dermal progenitor cells originating from the somites. Development 127, 2155–2164.

Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F., Bellard, M., 1997. ladybird, a tandem of homeobox genes that maintain late wingless expression in terminal and dorsal epidermis of the Drosophila embryo. Development 124, 91–100.

Jagla, T., Bellard, F., Lutz, Y., Dretzen, G., Bellard, M., Jagla, K., 1998. ladybird determines cell fate decisions during diversification of Drosophila somatic muscles. Development 125, 3699–3708.

Jagla, T., Bellard, F., Vonesch, J.L., Bellard, M., Dastugue, B., Jagla, K., 1999. Plasticity within the lateral somatic mesoderm of Drosophila embryos. Int. J. Dev. Biol. 43, 571–573.

Jagla, K., Bellard, M., Frasch, M., 2001. A cluster of Drosophila homeobox genes involved in mesoderm differentiation programs. BioEssays 23, 125–133.

Jékely, G., Arendt, D., 2007. Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy. BioTechniques 42, 751–755.

Kanamoto, T., Terada, K., Yoshikawa, H., Furukawa, T., 2006. Cloning and expression pattern of lbx3, a novel chick homeobox gene. Gene Expr. Patterns 6, 241–246.

Knirr, S., Azpiazu, N., Frasch, M., 1999. The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. Development 126, 4525–4535.

Kruger, M., Schafer, K., Braun, T., 2002. The homeobox containing gene Lbx1 is required for correct dorsal–ventral patterning of the neural tube. J. Neurochem. 82, 774–782.

Larroux, C., Fahey, B., Degnan, S.M., Adamski, M., Rokhsar, D.S., Degnan, B.M., 2007. The NK homeobox gene cluster predates the origin of Hox genes. Curr. Biol. 17, 706–710.

Larsen, C.W., Hirst, E., Alexandre, C., Vincent, J.P., 2003. Segment boundary formation in Drosophila embryos. Development 130, 5625–5635.

Lee, K.H., Xu, Q., Breitbart, R.E., 1996. A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. Dev. Biol. 180, 722–731.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I., Harvey, R.P., 1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119, 419–431.

Logan, C., Wingate, R.J., McKay, I.J., Lumsden, A., 1998. Tlx-1 and Tlx-3 homeobox gene expression in cranial sensory ganglia and hindbrain of the chick embryo: markers of patterned connectivity. J. Neurosci. 18, 5389–5402.

Lowe, C.J., Wu, M., Salic, A., Evans, L., Lander, E., Stange-Thomann, N., Gruber, C.E., Gerhart, J., Kirschner, M., 2003. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113, 853–865.

Lowe, C.J., Terasaki, M., Wu, M., Freeman, R.M., Runft, L., Kwan, K., Haigo, S., Aronowicz, J., Lander, E., Gruber, C., Smith, M., Kirschner, M., Gerhart, J., 2006. Dorsoventral patterning in hemichordates: insights into early chordate evolution. PLoS Biol. 4, e291.

Luke, G.N., Castro, L.F., McLay, K., Bird, C., Coulson, A., Holland, P.W., 2003. Dispersal of NK homeobox gene clusters in amphioxus and humans. Proc. Natl. Acad. Sci. U. S. A. 100, 5292–5295.

Luke, G.N. The Amphioxus NK Cluster. Thesis, Univ. Reading, UK (2004).

Master, V.A., Kourakis, M.J., Martindale, M.Q., 1996. Isolation, characterization, and expression of Le-msx, a maternally expressed member of the msx gene family from the glossiphoniid leech, Helobdella. Dev. Dyn. 207, 404–419.

Minelli, A., Fusco, G., 2004. Evo-devo perspectives on segmentation: model organisms, and beyond. Trends Ecol. Evol. 19, 423–429.

Minoo, P., Su, G., Drum, H., Bringas, P., Kimura, S., 1999. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(−/−) mouse embryos. Dev. Biol. 209, 60–71.

Nakao, T., 1974. An electron microscopic study of the circulatory system in *Nereis japonica*. J. Morphol. 144, 217–235.

Nardelli-Haefliger, D., Shankland, M., 1993. Lox10, a member of the NK-2 homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech Helobdella. Development 118, 877–892.

Newman, C.S., Krieg, P.A., 1999. The Xenopus bagpipe-related homeobox gene zampogna is expressed in the pharyngeal endoderm and the visceral musculature of the midgut. Dev. Genes Evol. 209, 132–134.

Newman, C.S., Grow, M.W., Cleaver, O., Chia, F., Krieg, P., 1997. Xbap, a vertebrate gene related to bagpipe, is expressed in developing craniofacial structures and in anterior gut muscle. Dev. Biol. 181, 223–233.

Newman, C.S., Reecy, J., Grow, M.W., Ni, K., Boettger, T., Kessel, M., Schwartz, R.J., Krieg, P.A., 2000. Transient cardiac expression of the tinman-family homeobox gene, XNkx2–10. Mech. Dev. 91, 369–373.

Nikolova, M., Chen, X., Lufkin, T., 1997. Nkx2.6 expression is transiently and specifically restricted to the branchial region of pharyngeal-stage mouse embryos. Mech. Dev. 69, 215–218.

Nose, A., Isshiki, T., Takeichi, M., 1998. Regional specification of muscle progenitors in Drosophila: the role of the msh homeobox gene. Development 125, 215–223.

Pabst, O., Schneider, A., Brand, T., Arnold, H.H., 1997. The mouse Nkx2–3 homeodomain gene is expressed in gut mesenchyme during pre- and postnatal mouse development. Dev. Dyn. 209, 29–35.

Patel, N.H., 2003. The ancestry of segmentation. Dev. Cell 5, 2–4.

Peel, A., Akam, M., 2003. Evolution of segmentation: rolling back the clock. Curr. Biol. 13, R708–R710.

Pennisi, E., Roush, W., 1997. Developing a new view of evolution. Science 277, 34–37.

Pollard, S.L., Holland, P.W., 2000. Evidence for 14 homeobox gene clusters in human genome ancestry. Curr. Biol. 10, 1059–1062.

Prud'homme, B., de Rosa, R., Arendt, D., Julien, J.F., Pajaziti, R., Dorresteijn, A.W., Adoutte, A., Wittbrodt, J., Balavoine, G., 2003. Arthropod-like expression patterns of engrailed and wingless in the annelid Platynereis dumerilii suggest a role in segment formation. Curr. Biol. 13, 1876–1881.

Qian, Y., Shirasawa, S., Chen, C.L., Cheng, L., Ma, Q., 2002. Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. Genes Dev. 16, 1220–1233.

Raible, F., Tessmar-Raible, K., Osoegawa, K., Wincker, P., Jubin, C., Balavoine, G., Ferrier, D., Benes, V., de Jong, P., Weissenbach, J., Bork, P., Arendt, D., 2005. Vertebrate-type intron-rich genes in the marine annelid Platynereis dumerilii. Science 310, 1325–1326.

Raju, K., Tang, S., Dube, I.D., Kamel-Reid, S., Bryce, D.M., Breitman, M.L., 1993. Characterization and developmental expression of Tlx-1, the murine homolog of HOX11. Mech. Dev. 44, 51–64.

Rida, P.C., Le Minh, N., Jiang, Y.J., 2004. A Notch feeling of somite segmentation and beyond. Dev. Biol. 265, 2–22.

Schneider, A., Mijalski, T., Schlange, T., Dai, W., Overbeek, P., Arnold, H.H., Brand, T., 1999. The homeobox gene NKX3.2 is a target of left–right signalling and is expressed on opposite sides in chick and mouse embryos. Curr. Biol. 9, 911–914.

Schoppmeier, M., Damen, W.G., 2005. Suppressor of Hairless and Presenilin phenotypes imply involvement of canonical Notch-signalling in segmentation of the spider Cupiennius salei. Dev. Biol. 280, 211–224.

Schubert, F.R., Fainsod, A., Gruenbaum, Y., Gruss, P., 1995. Expression of the novel murine homeobox gene Sax-1 in the developing nervous system. Mech. Dev. 51, 99–114.

Schubert, F.R., Dietrich, S., Mootoosamy, R.C., Chapman, S.C., Lumsden, A., 2001. Lbx1 marks a subset of interneurons in chick hindbrain and spinal cord. Mech. Dev. 101, 181–185.

Seaver, E.C., 2003. Segmentation: mono- or polyphyletic? Int. J. Dev. Biol. 47, 583–595.

Seaver, E.C., Kaneshige, L.M., 2006. Expression of 'segmentation' genes during larval and juvenile development in the polychaetes Capitella sp. I and H. elegans. Dev. Biol. 289, 179–194.

Seaver, E.C., Shankland, M., 2001. Establishment of segment polarity in the ectoderm of the leech Helobdella. Development 128, 1629–1641.

Seaver, E.C., Paulson, D.A., Irvine, S.Q., Martindale, M.Q., 2001. The spatial and temporal expression of Ch-en, the engrailed gene in the polychaete Chaetopterus, does not support a role in body axis segmentation. Dev. Biol. 236, 195–209.

Simoes-Costa, M.S., Vasconcelos, M., Sampaio, A.C., Cravo, R.M., Linhares, V.L., Hochgreb, T., Yan, C.Y., Davidson, B., Xavier-Neto, J., 2005. The evolutionary origin of cardiac chambers. Dev. Biol. 277, 1–15.

Simon, R., Lufkin, T., 2003. Postnatal lethality in mice lacking the Sax2 homeobox gene homologous to Drosophila S59/slouch: evidence for positive and negative autoregulation. Mol. Cell. Biol. 23, 9046–9060.

Stollewerk, A., Schoppmeier, M., Damen, W.G., 2003. Involvement of Notch and Delta genes in spider segmentation. Nature 423, 863–865.

Tanaka, M., Lyons, G.E., Izumo, S., 1999. Expression of the Nkx3.1 homobox gene during pre and postnatal development. Mech. Dev. 85, 179–182.

Tanaka, M., Schinke, M., Liao, H.S., Yamasaki, N., Izumo, S., 2001. Nkx2.5 and Nkx2.6, homologs of Drosophila tinman, are required for development of the pharynx. Mol. Cell. Biol. 21, 4391–4398.

Tautz, D., 2004. Segmentation. Dev. Cell 7, 301–312.

Tessmar-Raible, K., Steinmetz, P.R., Snyman, H., Hassel, M., and Arendt, D. (2005). Fluorescent two-color whole mount in situ hybridization in Platynereis dumerilii (Polychaeta, Annelida), an emerging marine molecular model for evolution and development. BioTechniques 39, 460, 462, 464.

Tessmar-Raible, K., Raible, F., Christodoulou, F., Guy, K., Rembold, M., Hausen, H., Arendt, D., 2007. Conserved sensory–neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. Cell 129, 1389–1400.

Tonissen, K.F., Drysdale, T.A., Lints, T.J., Harvey, R.P., Krieg, P.A., 1994. XNkx-2.5, a Xenopus gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. Dev. Biol. 162, 325–328.

Tribioli, C., Frasch, M., Lufkin, T., 1997. Bapx1: an evolutionary conserved homologue of the Drosophila bagpipe homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. Mech. Dev. 65, 145–162.

Veraksa, A., Del Campo, M., McGinnis, W., 2000. Developmental patterning genes and their conserved functions: from model organisms to humans. Mol. Genet. Metab. 69, 85.

Wang, W., Lo, P., Frasch, M., Lufkin, T., 2000. Hmx: an evolutionary conserved homeobox gene family expressed in the developing nervous system in mice and Drosophila. Mech. Dev. 99, 123–137.

Zaffran, S., Das, G., Frasch, M., 2000. The NK-2 homeobox gene scarecrow (scro) is expressed in pharynx, ventral nerve cord and brain of Drosophila embryos. Mech. Dev. 94, 237–241.