

A 20535

# Development Genes and Evolution

217/6

2007



# Segmental expression of Pax3/7 and Engrailed homologs in tardigrade development

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Received: 20 December 2006 / Accepted: 28 March 2007  
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**Abstract** How morphological diversity arises through evolution of gene sequence is a major question in biology. In *Drosophila*, the genetic basis for body patterning and morphological segmentation has been studied intensively. It is clear that some of the genes in the *Drosophila* segmentation program are functioning similarly in certain other taxa, although many questions remain about when these gene functions arose and which taxa use these genes similarly to establish diverse body plans. Tardigrades are an outgroup to arthropods in the Ecdysozoa and, as such, can provide insight into how gene functions have evolved among the arthropods and their close relatives. We developed immunostaining methods for tardigrade embryos, and we used cross-reactive antibodies to investigate the expression of homologs of the pair-rule gene *paired* (Pax3/7) and the segment polarity gene *engrailed* in the tardigrade *Hypsibius dujardini*. We find that in *H. dujardini* embryos, Pax3/7 protein localizes not in a pair-rule pattern but in a segmentally iterated pattern, after the segments are established, in regions of the embryo where neurons later arise. Engrailed protein localizes in the posterior ectoderm of each segment before ectodermal segmentation is apparent. Together with previous results from others, our data support the conclusions that the pair-rule function of Pax3/7 is specific to the arthropods, that some of the ancient functions of Pax3/7 and Engrailed in ancestral bilaterians

may have been in neurogenesis, and that Engrailed may have a function in establishing morphological boundaries between segments that is conserved at least among the Panarthropoda.

**Keywords** Engrailed · Pax3/7 · Ecdysozoa · Segmentation · Development

## Introduction

How the genes that control morphology are modified through evolution to produce morphological diversity is a central question in biology. Development of a segmented body plan is a process that occurs in diverse taxa, but the extent to which the known developmental cascades function in the same manner to control this process in different metazoans is not yet clear (see Budd 2001; Seaver 2003; Minelli and Fusco 2004; Tautz 2004 for review). In *Drosophila*, segmentation of the body axis is regulated by a cascade of gene activities in which maternal-effect, gap, pair-rule, and segment polarity, and Hox genes progressively pattern the embryo (Nüsslein-Volhard and Wieschaus 1980; see Tautz 2004 for review). We are interested in understanding when these patterning mechanisms evolved and in how they are used in forming different body plans throughout evolution.

In the *Drosophila* segmentation pathway, pair-rule genes are the first genes to be expressed in a segmentally iterated pattern. Before gastrulation, pair-rule genes are expressed in alternating segments of *Drosophila* embryos, and when their functions are disrupted, the even- or odd-numbered segments fail to develop properly (Nüsslein-Volhard and Wieschaus 1980; reviewed in Davis and Patel 2003). The pair-rule genes include *paired* (*prd*), *hairy*, *runt*, *fushi-*

Communicated by S. Roth

**Electronic supplementary material** The online version of this article (doi:10.1007/s00427-007-0152-5) contains supplementary material, which is available to authorized users.

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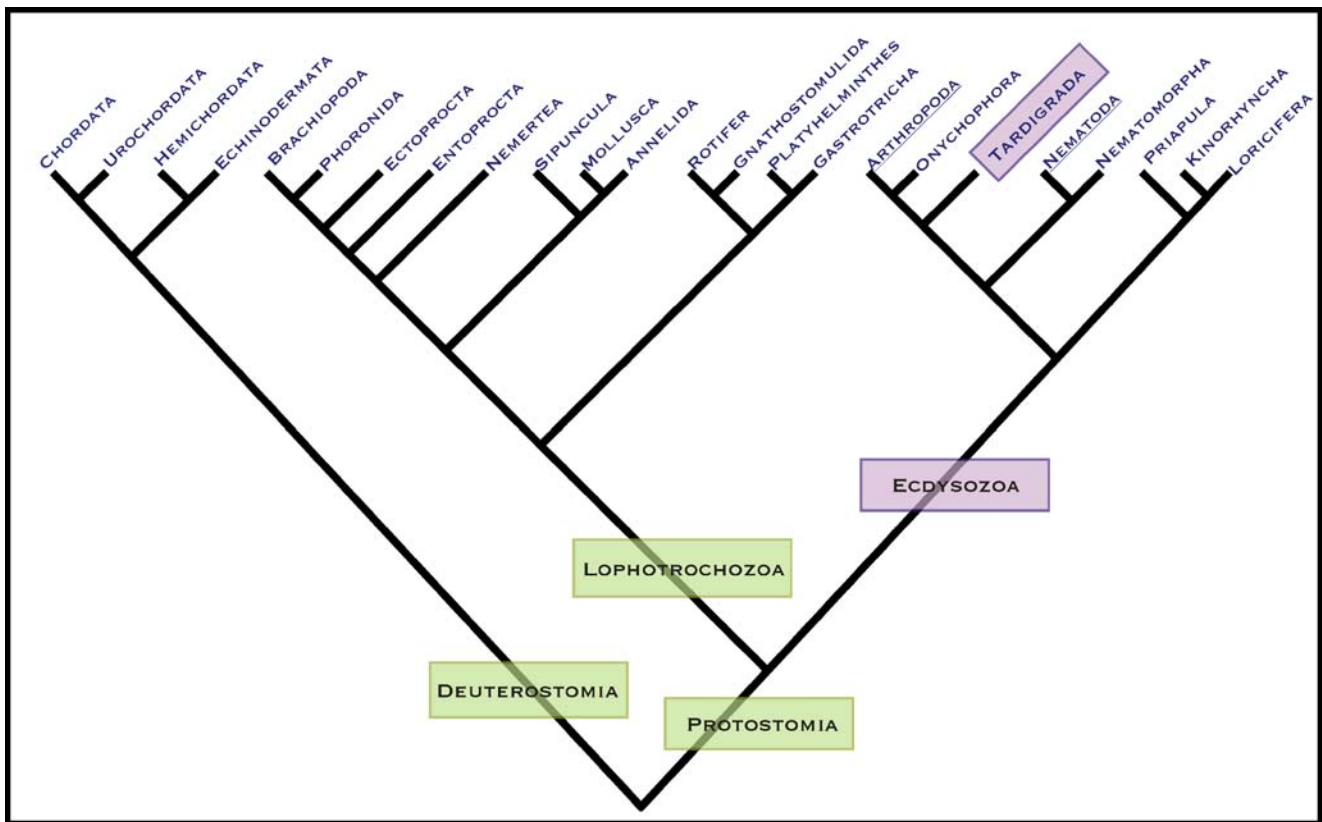
*tarazu* (*ftz*), and *even-skipped* (*eve*). Nearly all of the known pair-rule genes encode transcription factors; for example, *prd* encodes a Pax3/7-class transcription factor with a paired domain and a homeodomain. After gastrulation, *prd*, *runt*, and *eve* are transiently expressed in every segment, along with segment polarity genes (Kilchherr et al. 1986; Macdonald et al. 1986; Frasch et al. 1987). This pattern of pair-rule gene expression is conserved among several other holometabolous insects (flies, fleas, moths, bees, and beetles; reviewed in Davis and Patel 2003). Some pair-rule genes are transiently expressed with a two-segment periodicity in centipedes and mites (reviewed in Davis and Patel 2003), suggesting that pair-rule patterning may have existed early in arthropod evolution or arose multiple times within the arthropod clade. To our knowledge, there has been no substantiated report of pair-rule patterning outside of the arthropods. As a result, whether pair-rule patterning existed before the evolution of the arthropods and exists in close relatives of the arthropods or whether it did not arise until after evolution of the arthropods is not yet clear.

Segment polarity genes are expressed in stripes with a single segment periodicity in *Drosophila* (Ingham 1991). The segment polarity protein Engrailed, which encodes a homeodomain-containing transcription factor, is initially expressed in ectodermal stripes in the posterior compartment of every developing segment, where it is necessary for differentiation of that region of the segment and for establishing morphological separations between segments (Nüsslein-Volhard and Wieschaus 1980; Kornberg 1981; DiNardo et al. 1985; Larsen et al. 2003). Engrailed is later expressed in neuroblasts, where it specifies cell fates (Bhat and Schedl 1997). The expression pattern of *engrailed* in embryos of diverse arthropods suggests that early arthropods used *engrailed* in segmental patterning and neurogenesis (Patel et al. 1989; Manzanares et al. 1993; Hughes and Kaufman 2002), but the function of *engrailed* in segmentation outside of the arthropods is less clear. In onychophorans (Wedeen et al. 1997; Eriksson et al. 2005), a mollusc (Jacobs et al. 2000), and several annelids (Wedeen and Weisblat 1991; Shain et al. 2000; Bely and Wray 2001; Seaver et al. 2001), *engrailed* is expressed in segmentally iterated stripes but not until after the segments are morphologically apparent. Eliminating *engrailed*-expressing cells in a leech does not affect overt segment differentiation (Shain et al. 2000; Seaver and Shankland 2001). These data suggest that *engrailed* does not establish morphological separations between the segments outside of the arthropods. Conflicting data exists in another annelid: In *Platynereis dumerilii*, *engrailed* has a segment polarity expression pattern before segments can be morphologically detected during larval development, but it is unclear whether this is during a stage of segmental development

that is homologous to segmentation in arthropods or ancestral annelids (Prud'homme et al. 2003). Later in *Platynereis* development, at a stage at which segmentation occurs by a process more similar to segmentation in arthropods, *engrailed* is expressed at the anterior of each developing larval segment before segments can be morphologically detected (Prud'homme et al. 2003). Whether *engrailed* functions to direct segment morphogenesis in *Platynereis* and whether its expression pattern reflects conserved roles or convergent deployment of this gene in segmentation are not yet clear, leaving some question as to whether the role of *engrailed* in establishing separations between segments is conserved outside of the arthropods.

The arthropods, onychophorans, and tardigrades comprise the superphylum Panarthropoda (Nielsen 1995; Garey 2001; Halanych 2004). Tardigrades are likely to be the sister phylum to the arthropod–onychophoran clade (Garey et al. 1999; Fig. 1) and, as such, may provide insight into when specific segmentation gene functions found in *Drosophila* first evolved.

To further address how the segmental patterning cascade functions outside of arthropods, we have developed an immunostaining protocol for embryos of the tardigrade *Hypsibius dujardini*. In this study, we report the first immunolocalization patterns in this phylum. Cross-reactive antibodies have been invaluable tools in studying the evolution of segmentation gene expression (Patel et al. 1989; Davis et al. 2005). We have used existing cross-reactive antibodies to Engrailed (a segment polarity gene product) and to Pax3/7-class proteins (which in *Drosophila* are encoded by the pair rule gene *prd* as well as *gooseberry* and *gooseberry-neuro*) to investigate their localization in embryos of *H. dujardini*. We report that these proteins localize in segmentally iterated patterns in the ectoderm and that Pax3/7 is not found in a pair-rule pattern during any stage of embryogenesis. We find that the segmentally iterated expression of Pax3/7 and Engrailed homologs arises after segmentation of the endomesoderm is first evident in multiplane differential interference contrast (DIC) recordings of *H. dujardini* embryogenesis but before the ectoderm is visibly segmented. The expression patterns of these genes is thus the earliest detectable sign of segmental organization in the ectoderm. The results suggest that Engrailed might play a role in establishing morphological segmental separations in the ectoderm in tardigrades, as it does in *Drosophila*. We suggest that the pair-rule function of Pax3/7 may have arisen near the base of arthropod evolution or within specific arthropod groups, after the arthropods and onychophorans diverged from the tardigrade lineage, and that the role of Engrailed in segment morphogenesis in the ectoderm may be conserved within the Panarthropoda.



**Fig. 1** Evolutionary position of tardigrades based on 18s rRNA data from Garey et al. (1999) and Garey (2001). Tardigrades are members of the Ecdysozoa

## Materials and methods

### Culture and embryo collection

*H. dujardini* were obtained commercially from Sciento (Manchester, UK) and were cultured at room temperature (RT) in commercial bottled spring water (Crystal Geysir or Deer Park) in 60-mm glass Petri plates. Species identity was confirmed by comparison to the description of *H. dujardini* in Pilato et al. 2006. Cultures were cleaned by several changes of spring water and supplied with fresh *Chlorrococcum* sp. algal culture (about 4:1 vol cleaned tardigrade culture: *Chlorrococcum* culture) as a food source every 10 days. *Chlorrococcum* cultures were obtained from Sciento. *H. dujardini* is parthenogenetic and produces embryos every 5 days, at which time embryos undergoing meiosis are deposited in the exuvia of the molting adult. Embryos were collected by removing each exuvia with a pair of 25-gauge surgical needles, using a scissor-like motion to cut the exuvia. Approximately 100 embryos could be collected in this manner in an hour.

### Time-lapse recordings

For imaging, *H. dujardini* embryos were mounted in their exuvia on uncoated glass microscope slides in bottled

spring water. 4D DIC microscopy was carried out using a C2400-07 Hamamatsu Newvicon video camera (Hamamatsu Photonics) mounted on a Nikon Eclipse 800 microscope (Nikon Instrument Group). Images were acquired at 1- $\mu$ m optical sections every 2–5 min during embryogenesis and analyzed with Metamorph v. 6.3r5 (Molecular Devices).

### Scanning electron microscopy

Tardigrades were fixed in 2% glutaraldehyde in spring water. Samples were stored at 4°C overnight or for several days before processing. After several rinses in spring water, the tardigrades were post-fixed in 1% osmium tetroxide for 1 h and dehydrated through an increasing series of ethanols (30, 50, 75, 90, 100, and 100%). The samples were transferred in absolute ethanol to a Balzers CPD 020 critical point dryer (Balzers Union, Liechtenstein) and dried using liquid carbon dioxide as the transition solvent. Tardigrades were mounted onto aluminum scanning electron microscopy stubs with double-sided carbon adhesive tabs and sputter coated with gold/palladium alloy (60:40) to a thickness of 20 nm using a Hummer X Sputter Coater (Anatech, Alexandria, VA). Specimens were viewed on a Cambridge Stereoscan S200 scanning electron

microscope using an accelerating voltage of 20 kV, working distance of 25 mm, and specimen tilt of 40° (LEO Electron Microscopy, Thornwood, NY).

#### Alkaline phosphatase staining

Embryos were removed from the parental exuvia and fixed for 20 min in 4% paraformaldehyde in 0.5× phosphate-buffered saline (PBS)+Triton X-100 (0.5× PBT). Sonication in paraformaldehyde was carried out on a Branson 250 Sonifier using four pulses of 5 s each at an amplitude of 2.2 with a constant duty cycle, with 15 s recovery on ice between pulses. Sonicated embryos were allowed to recover for 15 min on ice and then rinsed two times in alkaline phosphatase staining buffer that was prepared according to the protocol provided in Imai et al. (2000). Alkaline phosphatase staining was then carried out following the protocol of Imai et al. (2000).

#### Fixation and immunohistochemistry

After removal of the parental exuvia, embryos were treated with 5 U/ml chitinase (Sigma), 10 mg/ml chymotrypsin (Sigma) in 0.5× PBS for 1 h at RT, washed three times for 5 min each in spring water, and then fixed for 20 min in absolute methanol at 4°C, followed by a 90–70–50% methanol series for 5 min each at RT. Embryos were then post-fixed for 10 min at RT in 4% paraformaldehyde in 0.5× PBT. Sonication in paraformaldehyde was carried out on a Branson 250 Sonifier using four pulses of 5 s each at an amplitude of 2.2 with a constant duty cycle, with 15 s recovery on ice between pulses. Sonicated embryos were allowed to recover for 15 min on ice and then were washed five times for 5 min each in 0.5× PBT. Embryos were blocked in 5% dimethyl sulfoxide and 1% bovine serum albumin in 0.5× PBT for 15 min followed by overnight incubation at 4°C with primary antibodies (mAb4F11, mAbDP311, mAbDP312, or anti-HRP [Jackson ImmunoResearch] at 1:10 plus rabbit anti-actin [Cytoskeleton] at 1:10–1:25) in blocking solution. The next day, embryos were washed three times for 5 min each and two times for 20 min each in 0.5× PBT followed by addition of secondary antibodies for 1 h at RT. Embryos were then washed three times for 5 min each and two times for 20 min each in 0.5× PBT. A tyramide amplification system (Molecular Probes) was used to amplify the signal for mAb4F11, mAbDP311, and mAbDP312. Embryos were then washed three times for 5 min each in 0.5× PBT, treated with 4',6-diamidino-2-phenylindole (DAPI; 5 µg/µl) and topro-3 (2 µM) for 20 min in 0.5× PBT, washed three times for 5 min in 0.5× PBT, and mounted on gelatin-subbed slides in mounting media (90% glycerol, 20 mM Tris pH 8.0, and 0.5% *n*-propyl gallate). Imaging was conducted

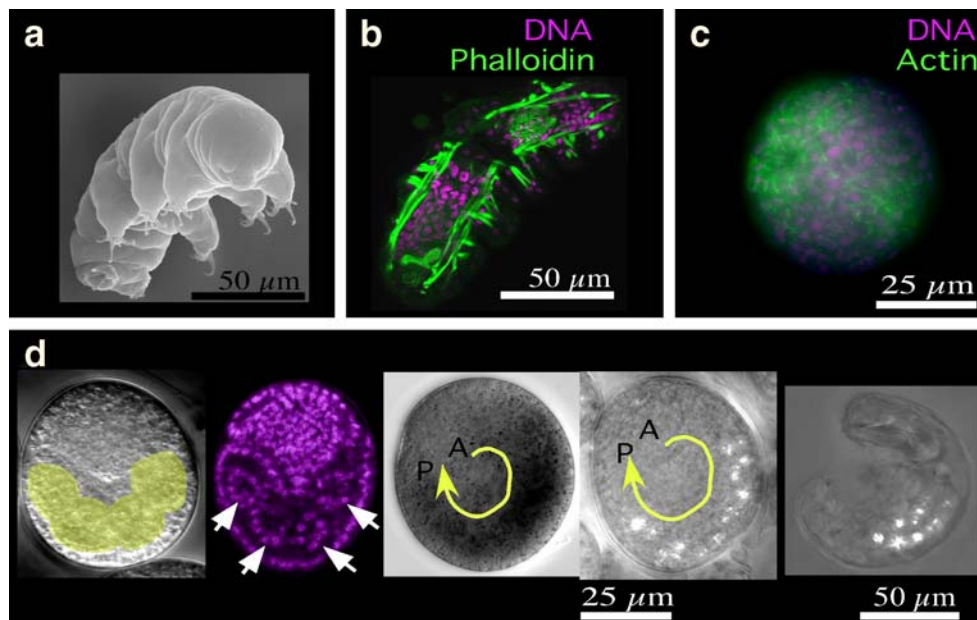
on a Zeiss LSM510 laser scanning confocal microscope. Approximately 20% of the embryos collected for each immunostaining assay remain intact during this protocol and are rendered permeable to antibodies. It was confirmed that all cells in the embryos were permeable to antibodies by using antibodies to actin and tubulin (mouse anti- $\alpha$ -tubulin, Sigma) alongside the antibodies to Engrailed and Pax3/7 group proteins. The stage of embryonic development of fixed embryos was determined based on a staging series of the appearance of morphological characters from 4D DIC microscopy of live embryos (Gabriel et al. in preparation). A total of 3–23 fixed embryos were examined for each stage of immunolocalization described in this paper.

## Results

Endomesodermal pouches are the earliest morphological evidence of segmentation in *H. dujardini* embryos

Upon hatching, tardigrades have a visibly segmented external cuticle with four repeating limb-bearing units, and muscle and ganglia are segmentally repeated (Marcus 1929). We have confirmed this body organization in *H. dujardini* by scanning electron microscopy (Fig. 2a) and by phalloidin staining of adults to observe the segmentally repeated pattern of developing muscles (Fig. 2b). To begin to identify when segmentation is initiated, we produced multiplane time-lapse DIC recordings of embryogenesis and examined them for evidence of segmentation. In *H. dujardini*, the earliest evidence of morphological segmentation that we have found is the constriction of the posterior portion of a tube of cells along the midline, originating from the proctodaeum, to form four pouches, starting approximately 24 h after the completion of meiosis (hpm; Fig. 2d, S7). DAPI staining of embryos at this stage shows 20–30 nuclei in each pouch (Fig. 2d, center left panel). Our findings are consistent with those of Marcus (1929), who described coelomic pouches forming from the archenteron, and with those of Eibye-Jacobsen (1996), who described the presence of similar pouches of cells in the posterior of an embryo from transmission electron micrographs.

We examined complete DIC optical sections of embryos at this stage and found that the pouches in *H. dujardini* arise as midline structures, such that the entire posterior portion of the midline tube of cells appears to become constricted into four spheres of cells. The hollow tube of cells along the midline reforms within hours of its original constriction, and the pouches appear to separate from the tube and form bilaterally paired structures that may not be continuous with the midline tube. We have not yet followed the pouch cells by 4D microscopy to determine their



**Fig. 2** Tardigrade morphology. **a** Scanning electron micrograph of *Hypsibius dujardini* adult. **b** Adult tardigrade stained with phalloidin to mark musculature. **c** Embryo stained with an antibody to actin. **d** Formation of endomesodermal pouches. *Left panel*: embryo 26.5 h post-meiosis, after pouches have formed; pouches are highlighted in yellow. *Center left panel*: DAPI-stained embryo showing endomesodermal pouches (arrows). *Center panel*: DIC image of embryo stained

for alkaline phosphatase (AP) activity, yellow arrow marks A–P axis. *Right center panel*: Polarized light image of embryo 40–50 h post-meiosis (hpm); birefringent granules are visible in cells deriving from the segmented pouches; yellow arrow marks A–P axis. *Right panel*: Polarized light image of juvenile tardigrade showing birefringent granules in its morphologically distinct gut

ultimate fate, but Hejnl and Schnabel (2005) described similar pouches as somite precursors in the tardigrade *Thulinia stephaniae*. We have additional evidence to suggest that at least some of the pouch cells are likely to be endodermal precursors. Under polarized light, we observed birefringent granules in the cells of these pouches, after they have formed paired structures (Fig. 2d, center right panel), that are similar to birefringent granules found in gut cells in *C. elegans*. In *C. elegans*, these granules are specific to the gut and are known to be produced as a product of tryptophan catabolism (Siddiqui and Babu 1980). These granules can also be identified in endodermal cells of newly hatched *H. dujardini* juveniles using polarized light (Fig. 2d, right panel). To confirm the endodermal identity of these pouch cells, we carried out staining for activity of alkaline phosphatase (AP), an enzyme that is commonly used as a marker for developing endoderm (see Whittaker 1977; Freeman 2003 for examples). We find that AP staining marks the cells of the midline pouches, suggesting that at least some of the cells in the midline pouches are endodermal precursors. It is also probable that some of the cells in these pouches are somite precursors, as was described by Marcus (1929) and Hejnl and Schnabel (2005), as these authors followed the fate of the pouch cells farther in development than we have. We conclude that segmentation is evident as early as 24 h after meiosis in *H. dujardini* development, in the form of internal, segmentally iterated endomesoderm structures.

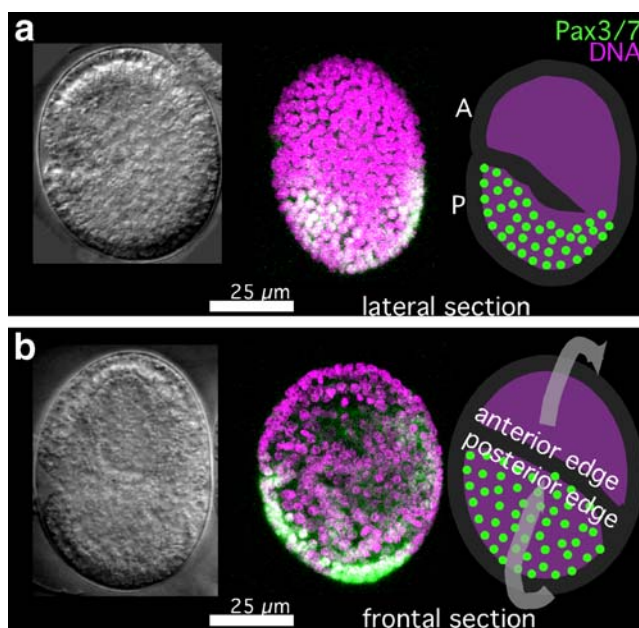
Pax3/7 expression is first evident in the posterior of the embryo after elongation

We developed an immunostaining protocol for *H. dujardini* embryos (see “Materials and methods”). Embryos were collected from meiosis through hatching and were processed for immunostaining. We determined that embryos could be successfully permeabilized to antibodies at all stages of embryonic development, as confirmed by staining with antibodies to actin and tubulin (Fig. 2c and data not shown). We used monoclonal antibodies DP311 and DP312, antibodies to *Drosophila* Prd that recognize Pax3/7 group homologs widely among the Ecdysozoa (Davis et al. 2005), to examine the distribution of Pax3/7 homologous proteins in *H. dujardini*. The localization pattern detected by the two antibodies was indistinguishable from each other at all stages examined, in apparently repeatable patterns of specific cells at each stage, and localization was nuclear, as expected. We conclude that these antibodies most likely recognize Pax3/7 homologs in *H. dujardini*, and we refer to the patterns produced as Pax3/7 localization. Pax3/7 is first detected in the nuclei of 40–60 cells in the posterior half of tardigrade embryos in the first hour after elongation (the lengthening of the embryo along the A–P axis) is completed (22–25 hpm). A summary of developmental landmarks in *H. dujardini* embryos is provided in Fig. 6c. This localization is seen throughout dorsal and lateral but not ventral cells primarily of the

exterior cell layer (presumptive ectoderm) and a few cells of the layer interior to this in the posterior 50% of the embryo (Fig. 3; in this and following figures, representative sections are shown, and the whole staining pattern that was observed is diagrammed). Localization is in contiguous cells and does not appear to have any segment-specific pattern. This expression pattern disappears before the onset of morphological segmentation. Permeabilization of the embryo before this stage was confirmed by staining with actin and tubulin antibodies, suggesting that the absence of Pax3/7 staining at earlier stages most likely reflects little or no Pax3/7 expression.

The first segmentally iterated localization of Pax3/7 is in ectodermal cells along the ventral midline

After the formation of the endomesodermal pouches (26–27 hpm), Pax3/7 localization is detected in a segmentally iterated pattern in nuclei of bilateral pairs of groups of one to three cells on either side of the ventral midline of the embryo. These cells are ectodermal, and they are in register with each endomesodermal pouch (Fig. 4a,b, S1, S4). This



**Fig. 3** Approximately 23 h post-meiosis; early, posterior localization of Pax3/7. **a** Lateral view. **b** Frontal view. *Arrow in b* indicates anterior–posterior axis. DIC images on the *left* of panels **a** and **b** are of embryos at the same stage as fluorescent images in the *center*. Fluorescent images are each projections of several confocal sections of an individual embryo. Cartoons on the *right* are representations of combined confocal stacks of the embryos in the *middle panel* of both **a** and **b** that show the overall pattern of nuclei recognized by the Pax3/7 antibodies, although, because of the density of stained nuclei in these embryos, each individual antibody-marked cell is not specifically represented. Pax3/7 is localized to the nuclei of 40–60 cells in the two exterior-most cell layers (presumptive ectoderm) throughout the posterior 50% of the embryo, except for the ventral-most portion

pattern of Pax3/7 localization first appears after endomesodermal pouches have formed but before morphologically evident segments have formed in the ectoderm. At this stage, Pax3/7 is also detected in the anterior of the embryo in the developing head region in approximately 40 cells that surround the tube of pharynx and mouth precursors (Fig. 4a,b). As development proceeds, the number of cells along the ventral midline and in the developing head that are marked by the Pax3/7 antibodies increases (Fig. 4c,d, S2, S5).

Pax3/7 expression continues to be detected in four groups of cells along the ventral midline through approximately 50 hpm, at which time the approximate maximum number of cells is recognized by DP311 and DP312. At this stage, each of the Pax3/7-positive groups along the midline consists of six to ten cells (Fig. 4e,f). The position of each pair of limb buds is lateral to these groups of Pax3/7-expressing cells. Each of the four groups of midline cells lies within the boundaries of a separate segment, as determined by the segmental boundaries that are evident in the ectoderm at this time. At this stage, Pax3/7 expression is also seen in approximately 80 cells of the developing head (Fig. 4e,f). This expression pattern persists through the rest of embryonic development.

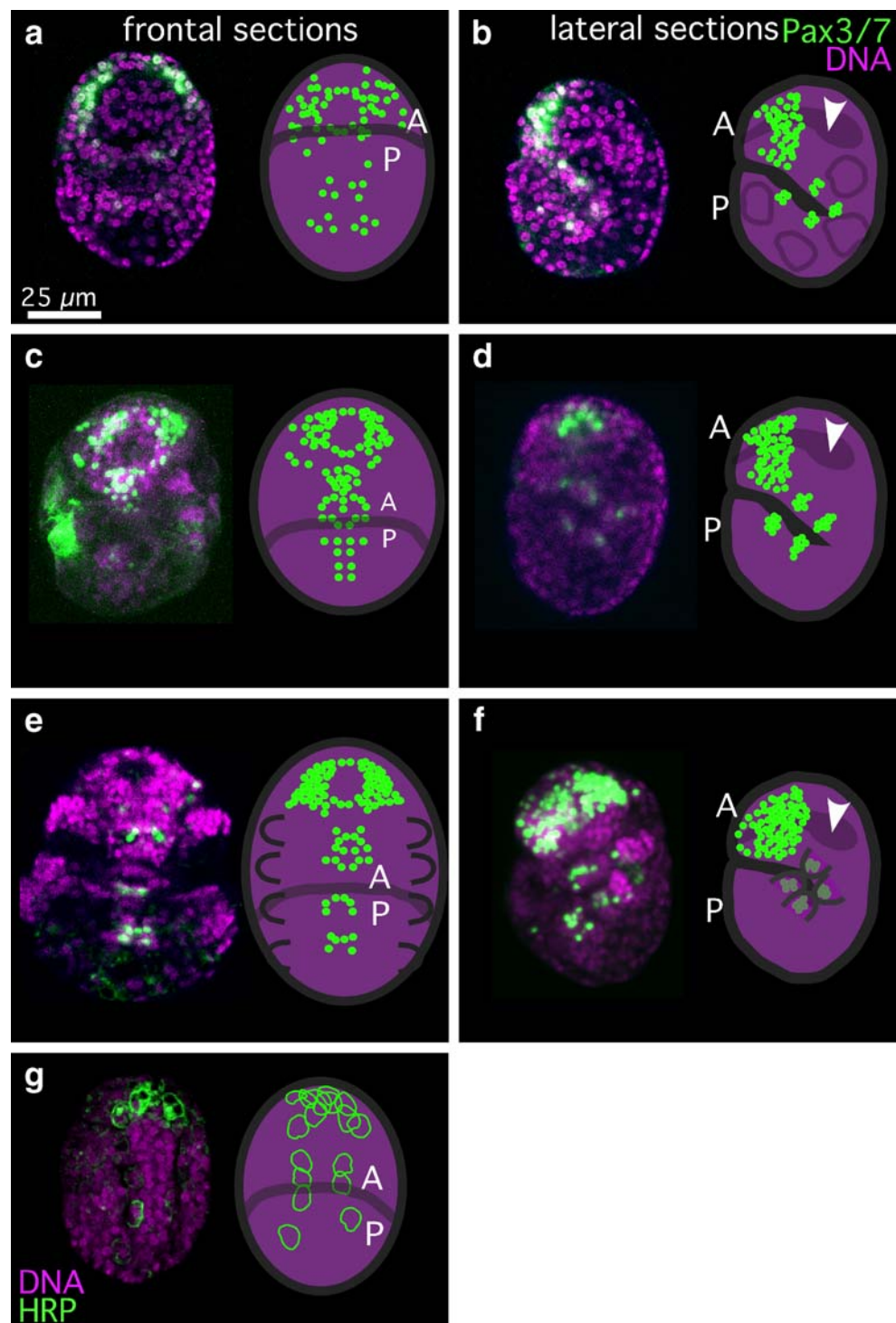
Because the developing head and the ventral midline are sites of the central nervous system (CNS) development (Marcus 1929; Hejnal and Schnabel 2005), we hypothesized that Pax3/7 expression might be in neuronal precursors. To test this hypothesis, we stained tardigrade embryos with an antibody to horseradish peroxidase (HRP), which recognizes plasma membrane glycoproteins in the developing neural tissue of a number of Ecdysozoans (Jan and Jan 1982; Haase et al. 2001). Although we were not able to double-stain embryos, cells with peripheral staining were found in regions expected to form neurons based on juvenile morphology, in the developing head and along the midline (Fig. 4g), at least some of which overlaps significantly with the region of Pax3/7 expression, suggesting that some or all of the Pax3/7-positive cells are likely to be neuronal precursors.

In summary, after *H. dujardini* embryos undergo elongation, Pax3/7 localizes throughout the posterior ectoderm except for the ventral-most portion. After this localization pattern disappears and segmentation of the endomesoderm is evident, Pax3/7 localizes in a segmentally associated pattern in cells that are likely neuronal precursors.

Engrailed localizes to ectodermal cells at the posterior of each developing segment before morphological segmentation of the ectoderm is evident

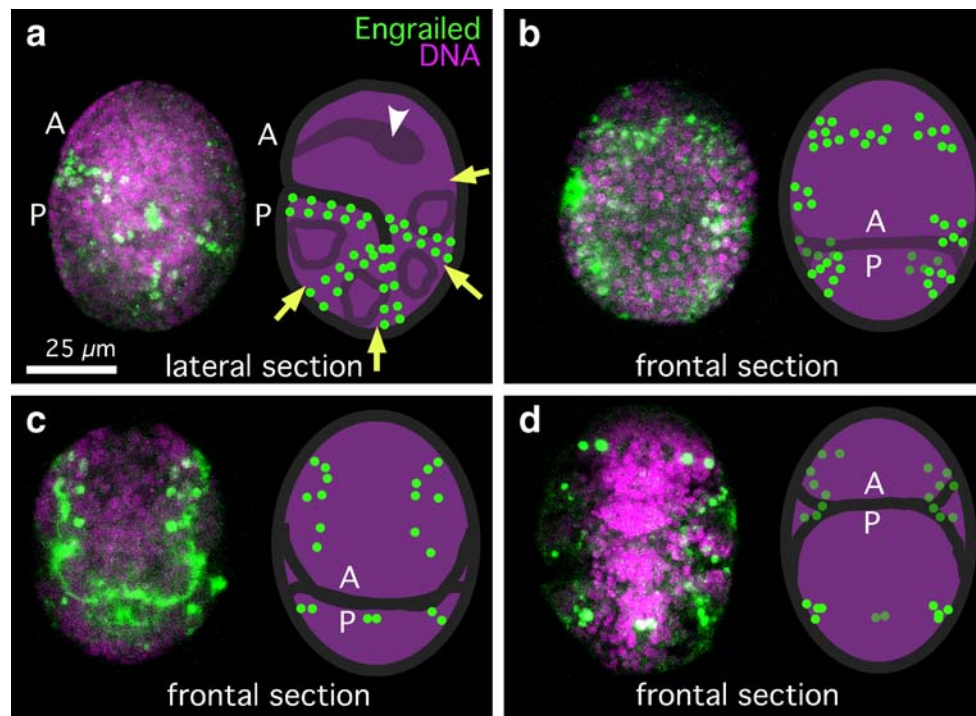
The monoclonal antibody 4F11 is an antibody to *Drosophila* Engrailed that recognizes Engrailed homologs

**Fig. 4** Localization of Pax3/7. In each panel, a projection of several confocal sections through the embryo is on the *left*, and a cartoon showing the positions of individual cells throughout the entire embryo is on the *right*. Boundaries of the embryo are represented in *gray*. Embryos are curled inside the eggshell after elongation occurs, with the ventral surface in the center of the embryo. In each cartoon, the anterior and posterior boundaries of the embryo are labeled (*A* and *P*). The *white arrowheads* in panels **b**, **d**, and **f** mark the developing pharynx. **a**, **b** Approximately 26 h post-meiosis. After endomesodermal segmentation is evident, Pax3/7 localization is detected in nuclei of four pairs of groups of one to three ectodermal cells on either side of the ventral midline and 40–59 nuclei in the developing head region. **c**, **d** Approximately 32 h post-meiosis. Pax3/7 localization is detected in nuclei of four pairs of groups of two to four ectodermal cells on either side of the ventral midline and 60–69 nuclei in the developing head region. **e**, **f** Approximately 50 h post-meiosis. Pax3/7 localization is detected in nuclei of four pairs of groups of three to five ectodermal cells on either side of the ventral midline between the limb buds and 70–80 nuclei in the developing head region. **g** Anti-HRP staining in cells of the developing head and along the midline



widely among Arthropoda (Patel et al. 1989). We used this antibody to examine the distribution of Engrailed homologs in *H. dujardini* embryos. The localization pattern detected by this antibody was in experimentally reproducible patterns at each stage and was nuclear, as expected. We conclude that this antibody most likely recognizes one or more Engrailed homologs in *H. dujardini*, and we refer to

the patterns produced as Engrailed localization. After endomesodermal pouch formation, Engrailed localizes in stripes of nuclei in the dorsal and lateral but not ventral-most portion of the ectoderm, in groups of eight to ten cells on either side of the midline, at the positions posterior to each underlying endomesodermal pouch (Fig. 5a,b, S3, S6). The posterior-most Engrailed-expressing cells are in



**Fig. 5** Engrailed localization during tardigrade embryogenesis. In each panel, a projection of several confocal sections through the embryo is on the *left*, and a cartoon showing the positions of individual cells throughout the entire embryo is on the *right*. Boundaries of the embryo are represented in *gray*. Embryos are curled inside the eggshell after elongation occurs, with the ventral surface in the center of the embryo. In each cartoon, the anterior and posterior boundaries of the embryo are labeled (*A* and *P*). Dimmed cells and text represent portions of the embryo that are not at the surface of the embryo that is facing the viewer. **a, b** Engrailed localization after endomesodermal pouch formation, approximately 26 hours post-meiosis. Engrailed

localizes to the dorsal ectoderm in the nuclei of stripes of eight to ten cells on each side of the midline at positions posterior to the underlying pouches. **a** Lateral view. Segmentally iterated endomesodermal pouches are represented by the *irregular gray pouches*. *Yellow arrows* indicate the sites where ectodermal furrows will form that mark the boundaries of developing segments, and the *white arrowhead* marks the developing pharynx. **b** Frontal view. **c, d** Engrailed localization during the rest of embryogenesis. From approximately 32 hours post-meiosis until hatching, Engrailed is localized to 20–30 cells in a bilaterally symmetric pattern throughout the embryo. **c** Approximately 32 h post-meiosis. **d** Approximately 50 h post-meiosis

the extreme posterior of the embryo, suggesting that these stripes are at the posterior-most portion of each segment. Morphological segmentation of the ectoderm is not evident when Engrailed first localizes in this pattern; both DIC and DAPI/topro-3 fluorescence imaging reveal a lawn of ectodermal nuclei with no apparent repeating units (Figs. 2d and 5b). The time at which this localization appears is concurrent with the onset of ventral and head Pax3/7 localization. Within 1–2 h after endomesodermal pouches have formed, segmentally iterated localization of Engrailed begins to disappear. Before the disappearance of this pattern of Engrailed localization, segmentally iterated furrows form in the ectoderm just posterior to the stripes of Engrailed. Unlike the pattern seen in *Drosophila* embryos, no transient pair-rule-like pattern of Engrailed localization is evident in *H. dujardini* embryos. Instead, we find that Engrailed localizes to all developing segmental units simultaneously.

Through the rest of embryogenesis, Engrailed localizes in a dynamic pattern

As segmentally iterated stripes of Engrailed disappear, Engrailed localization appears in 20–30 other cells in a bilaterally symmetric pattern throughout the embryo (Fig. 5). Initially, 14 cells in the anterior of the embryo and six cells in the posterior are marked by staining with the Engrailed antibody (Fig. 5c), and this increases to 16 cells in the anterior and ten cells in the posterior as development proceeds (Fig. 5d). Two of these cells appear to correspond to the posterior of the developing gut by comparison with DIC images of live embryos at the same stage of development. Except for these two cells, Engrailed localizes to cells at this stage that do not correspond to any as-yet discernible morphological features. This localization pattern persists through the rest of embryonic development.

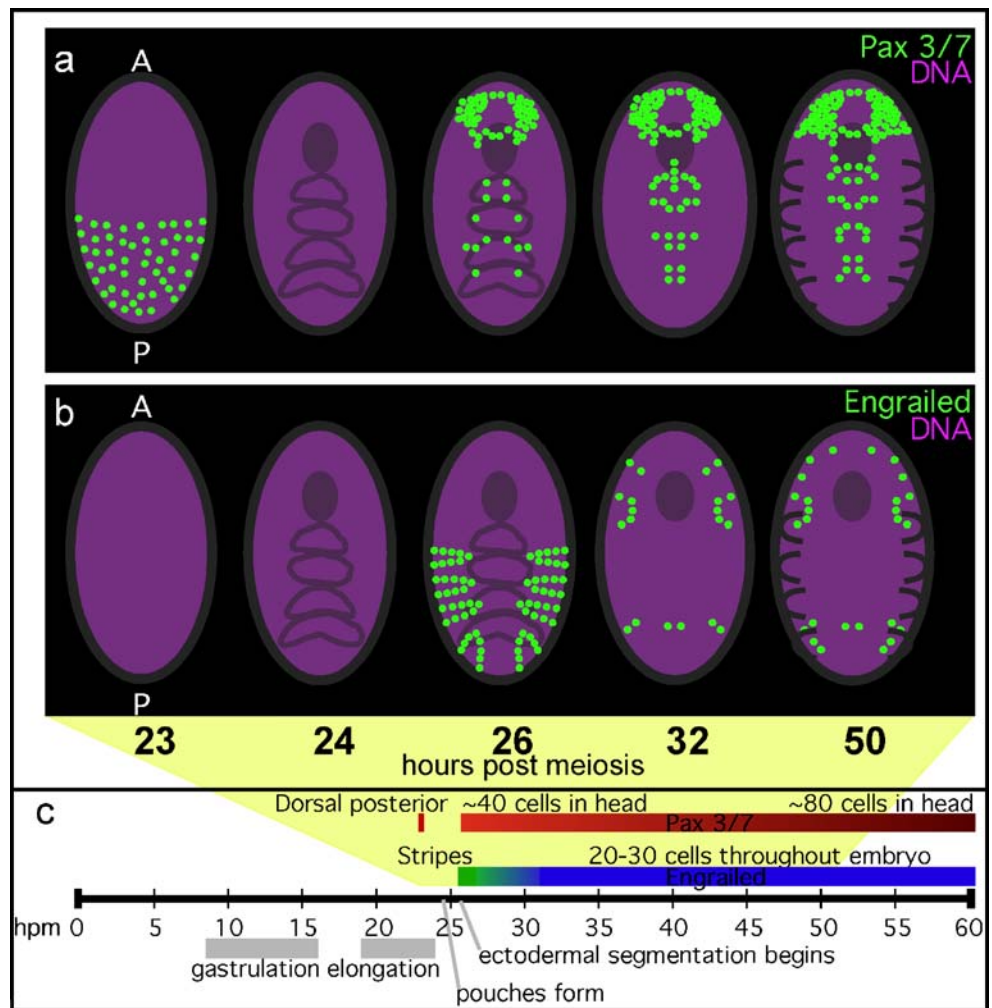
## Discussion

In this article, we report the first immunolocalization patterns in the phylum Tardigrada. The immunolocalization patterns produced by staining with antibodies to Engrailed and Pax3/7 homologs are associated with regions of the embryo where neuronal and segmental differentiation is occurring, similar to what has been seen in other species (Seaver 2003; Minelli and Fusco 2004; Tautz 2004 for review). In *H. dujardini* embryos, Pax3/7 localization is first detected after the elongation stage of embryogenesis in nuclei in the dorsal ectoderm in the posterior 50% of the embryo (Fig. 6a,c). This pattern of localization resembles the broad domains occupied by maternal factors in *Drosophila* (Nüsslein-Volhard and Wieschaus 1980), rather than the one- or two-segment periodicity that is most typical of Pax3/7 expression in arthropods, although transcripts at this stage of *H. dujardini* development are likely to be zygotic, rather than maternal. What role Pax3/7 may be playing at this stage is unknown.

Segmentally iterated Pax3/7 localization is first seen after the onset of segmentation of the endomesoderm, but before morphological segmentation of the ectoderm is evident in *H. dujardini* embryos. After segmented endomesodermal pouches have formed (26–27 hpm), Pax3/7 is detected in groups of ectodermal cells on either side of the ventral midline and in the developing head region. These groups of cells may correspond to sites of immunolocalization of anti-HRP, which marks neural development throughout the Ecdysozoa (Haase et al. 2001). This pattern of localization is consistent with a function in neural patterning that has been shown for Pax3/7 homologs in species as diverse as arthropods and mice (see Gutjahr et al. 1993; Koblar et al. 1999 for examples).

Pair-rule patterning has not been substantively demonstrated outside of the Arthropoda, and within the Arthropoda, not all groups may exhibit pair-rule patterning. In the grasshopper, *Schistocerca*, and the spider mite, *Tetranychus*, Pax3/7 appears in stripes of two-segment periodicity during at least some period of development

**Fig. 6** A summary of Pax3/7 and Engrailed expression throughout tardigrade embryogenesis. Cartoons in **a** and **b** represent embryos during 23–60 h post-meiosis, hpm). Times given are from a single representative DIC film of five synchronous embryos. **a, b** Representative drawings of Pax3/7 and Engrailed. Drawings are longitudinal views of embryos as they would appear if removed from the eggshell and uncurled that have been compiled from images of multiple embryos at each stage (for Pax3/7: 23 hpm,  $n=3$ ; 26 hpm,  $n=23$ ; 32 hpm,  $n=12$ ; 50 hpm,  $n=5$ ; for Engrailed: 26 hpm,  $n=18$ ; 32–50 hpm,  $n=9$ ). The staining pattern represents what is seen if all confocal sections of an embryo were superimposed on each other. **c** Timeline describing Pax3/7 and Engrailed localization during embryogenesis. Gray bars represent significant morphogenetic events. Red: Pax3/7; darker color represents increasing numbers of cells marked by DP311 and DP312 antibodies as development proceeds. Green: Engrailed in stripes. Blue: Engrailed in 20–30 cells during the rest of embryogenesis



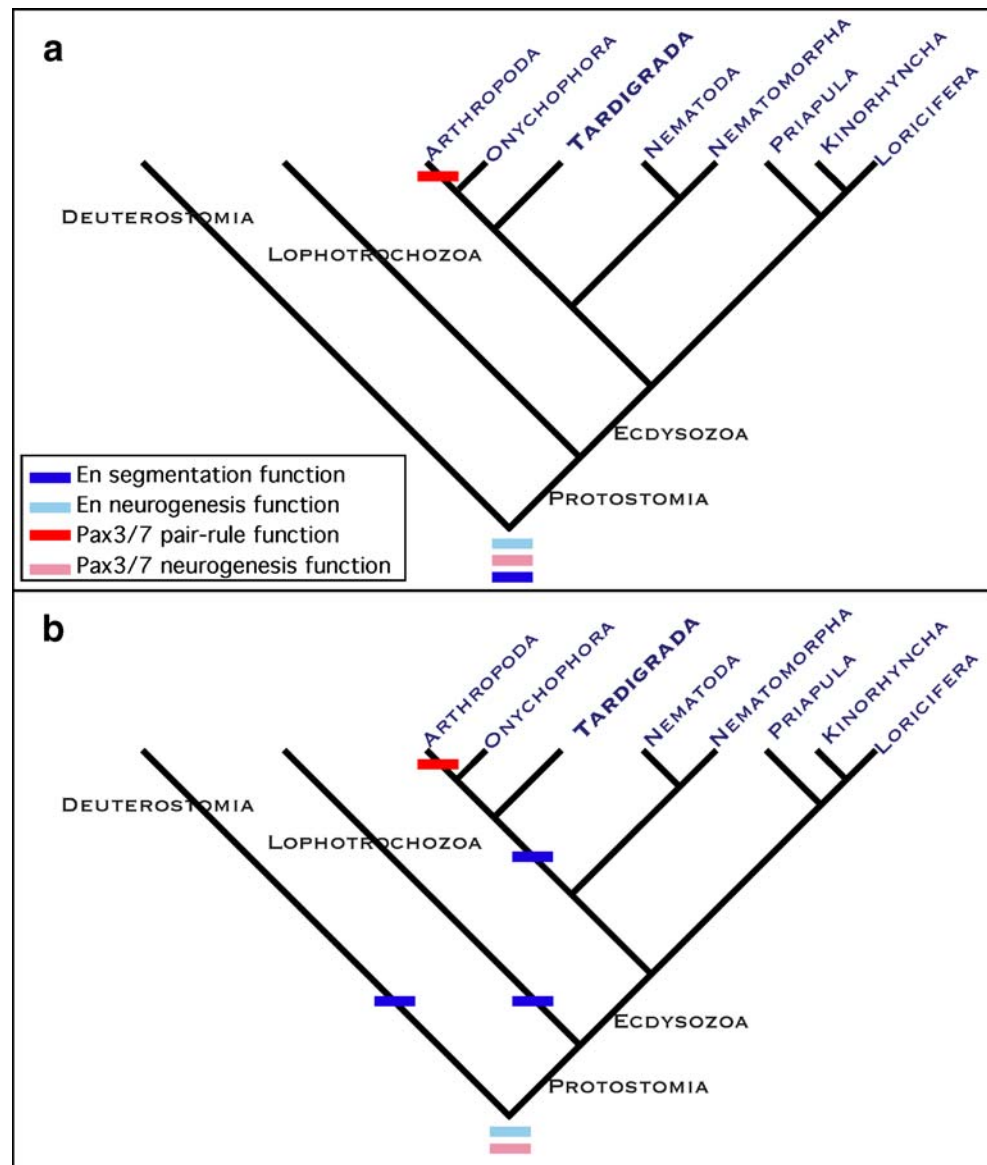
(Davis et al. 2001; Dearden et al. 2002), and in the centipede, *Strigamia maritima*, a pair-rule type expression pattern is seen for an *odd-skipped* family member and an ortholog of the gap gene *caudal* (Chipman et al. 2004). In all other arthropods where pair-rule gene expression has been investigated, these genes are expressed either in stripes in every developing segment, in broad posterior domains, or in a Hox-like domain (Patel et al. 1992; Dawes et al. 1994; Hughes and Kaufman 2002; Davis et al. 2005), although the periodicity of some expression patterns has yet to be resolved definitively.

To our knowledge, the only proposal of a pair-rule-like gene-patterning mechanism outside of the Arthropoda has been in zebrafish embryos, in which the *hairy* homolog *her1* was initially described as having a pair-rule expression pattern, in alternating somites (Müller et al. 1996). It now

appears, however, that this gene is expressed in every developing segment during some stage of somitogenesis, in an oscillating pattern that is controlled by Notch signaling, and loss of function of zebrafish hairy homologs do not show defects with strict two-segment periodicity (see Tautz 2004 for review).

Because tardigrades are close relatives of the arthropods and pair-rule patterning has not been demonstrated outside of the arthropods, our data support the theory that pair-rule patterning evolved within the arthropods, either once or multiple times (Fig. 7). To further test this hypothesis, it will, however, be necessary to determine if other pair-rule genes, such as *eve*, *ftz*, and *hairy*, or possibly divergent Pax3/7 homologs, are expressed in pair-rule patterns. The expression pattern reported here suggests that the most likely roles for Pax3/7 homologs in *H. dujardini* embryogenesis are in

**Fig. 7** Proposed evolution of Pax3/7 and Engrailed functions. Our results are consistent with the hypothesis that Pax3/7 and Engrailed functioned in neurogenesis in the common ancestor of protostomes and deuterostomes (*pink bars* for Pax3/7 and *light blue bars* for Engrailed), and that Pax3/7 homologs gained their pair-rule function near the time that the arthropods arose (*red bars*). Engrailed may have had a function in generating morphological separations between segments (*dark blue bars*) in the protostome–deuterostome ancestor (a), or this function of Engrailed may have arisen independently several times in metazoan evolution (b)



ectodermal patterning in the posterior and in patterning the CNS. It will be of interest to disrupt the function of Pax3/7 gene homologs in tardigrade embryos to determine if they are, indeed, playing roles in these developmental processes that are similar to the roles they play in other members of the Panarthropoda.

Engrailed is first recognized by MAb 4F11 in tardigrade embryos at the same stage as the segmentally associated Pax3/7 pattern is first seen (Fig. 6b,c). Engrailed localization appears in stripes in the ectoderm of tardigrade embryos that correspond to the posterior boundary of each developing segment. These stripes are first detected after endomesodermal segmentation has occurred but before the onset of ectodermal segmentation. In *Drosophila* segmentation, Engrailed is known to play a role in the formation of segmental boundaries in the ectoderm that is followed by segmentation of the endoderm and mesoderm (Baylies et al. 1995). Although segmentation of the endomesoderm is seen relatively earlier in *H. dujardini*, the localization of Engrailed to the posterior portion of future segments in the ectoderm, before the onset of morphological ectodermal segmentation, is similar to what is seen in *Drosophila* and other arthropods. This indicates that Engrailed might be playing a similar role in directing ectodermal segmentation in tardigrades as it plays in *Drosophila*. To determine if this localization pattern reflects a functional role in directing segmentation, it will be useful to disrupt the function of *engrailed* homologs in *H. dujardini* embryos to determine if segmentation of the ectoderm is, indeed, subsequently disrupted and, therefore, whether *engrailed* plays the same role in tardigrades as it does in arthropods.

Despite the fact that *engrailed* is expressed in developing segments of a number of diverse protostome species (Patel et al. 1989; Ingham 1991; Wedeen and Weisblat 1991; Manzanares et al. 1993; Wedeen et al. 1997; Jacobs et al. 2000; Shain et al. 2000; Bely and Wray 2001; Seaver et al. 2001; Hughes and Kaufman 2002; Prud'homme et al. 2003; Eriksson et al. 2005), it is unclear outside of the Arthropoda whether or not Engrailed is involved in directing the morphological separation of segment primordia before when those segments are morphologically evident. In one member of the Onychophora, Engrailed localizes to transient stripes in cells below the ectoderm concurrent with the first appearance of segmental structures (Wedeen et al. 1997). Wedeen et al. (1997) did not detect Engrailed in the ectoderm, suggesting either that this species has multiple *engrailed* homologs, some of which were not detected by the antibody used, or that Engrailed was localized in a segmentally iterated pattern in the mesoderm and ectoderm of the common ancestor of arthropods and onychophorans, and ectodermal localization was lost in onychophorans.

Our data are consistent with the hypothesis that Engrailed may play a conserved role in the formation of

segmental boundaries in the ectoderm of the Panarthropoda. It is possible either that Engrailed played this role in the last common bilaterian ancestor (Fig. 7a) or that Engrailed was co-opted for segmentation pathways multiple times in metazoan evolution (Fig. 7b). To distinguish between these possibilities, it will be useful to collect data on the function of *engrailed* in diverse groups by disrupting gene function, because understanding the function of Engrailed will tell us if it is, indeed, acting in a homologous fashion in the segmentation cascade. It will also be of interest to collect more data on the upstream activators of *engrailed* in tardigrades, to determine if the segmentation cascade is homologous within the Ecdysozoa. *prd* is a known upstream activator of *engrailed* in *Drosophila* (DiNardo and O'Farrell 1987). The localization patterns of Pax3/7 and Engrailed in *H. dujardini* embryos suggest that they do not interact in segmental patterning. At approximately 26 h post-meiosis, the only stage when Pax3/7 and Engrailed both localize in a segmentally iterated pattern, Pax3/7 is expressed along the midline of the ventral ectoderm, while Engrailed is expressed lateral to this and on the opposite side of the embryo, in the dorsal ectoderm (Fig. 6). Although Pax3/7 localizes to the dorsal ectoderm earlier in development, Engrailed is not detected here until after Pax3/7 disappears from the region (Fig. 6).

Besides its role in segmental patterning, *engrailed* plays a role in numerous other developmental processes, the most conserved of which is likely to be its role in neurogenesis. *Engrailed* is expressed in the developing nervous system of arthropods, annelids, chordates, and echinoderms, suggesting that an ancestral role of *engrailed* may have been in patterning the nervous system (Patel et al. 1989; Lowe and Wray 1997). A role in neural patterning is one possible explanation for the later, dynamic pattern of Engrailed localization that we describe here, although additional data is certainly required to confirm this speculation. Determining the role of *engrailed* in segmental patterning in nonarthropod Ecdysozoans will help to elucidate whether its function in initiating segmentation is evolutionarily conserved or if it functions in this way uniquely in the Arthropoda.

**Acknowledgments** We thank Greg Wray, Nipam Patel, and members of the Goldstein lab for discussions and comments on the manuscript and Nipam Patel for generously sharing cross-reactive antibodies. This work was funded by NSF grant IBN-0235658 to BG.

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