ORIGINAL ARTICLE

Identification and characterization of a *twist* ortholog in the polychaete annelid *Platynereis dumerilii* reveals mesodermal expression of *Pdu-twist*

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Abstract The basic helix-loop-helix transcription factor *twist* plays a key role during mesoderm development in Bilateria. In this study, we identified a *twist* ortholog in the polychaete annelid *Platynereis dumerilii* and analyze its expression during larval development, postlarval growth up to the adult stage, and caudal regeneration after amputation of posterior segments. At late larval stages, *Pdu-twist* is expressed in the mesodermal anlagen and in developing muscles. During adulthood and caudal regeneration, *Pdu-twist* is expressed in the posterior growth zone, in mesodermal cells within the newly forming segments and budding parapodia. Our results indicate that *Pdu-twist* is involved in mesoderm formation during larval development, posterior growth, and caudal regeneration.

Keywords Regeneration · Muscle · Transcription factor · Posterior growth · Segmentation

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Introduction

The mesoderm is a systematic characteristic of the Bilateria. It has been assumed that invention of the mesoderm facilitated the diversification of bilaterian body plan morphologies (Perez-Pomares and Munoz-Chapuli 2002). Several studies examining mesoderm development in species of the three major bilaterian groups, i.e., Deuterostomia, Ecdysozoa, and Lophotrochozoa, uncovered similar mechanisms of mesoderm formation (Technau and Scholz 2003). In the polychaete annelid *Platynereis dumerilii*, the mesoderm arises from cells that invaginate through the blastopore and accumulate between ectoderm and endoderm. During further development, these cells arrange into mesodermal packages in the three simultaneously formed anteriormost segments (Dorresteijn 1990). Starting from the larval nectochaete stage, segments are added continuously from a posterior subterminal proliferation zone throughout the entire life-span (Fischer and Dorresteijn 2004).

On the molecular level, a conserved regulator of mesoderm development is the gene *twist* (Technau and Scholz 2003). The basic helix-loop-helix (bHLH) transcription factor has been shown to be essential for mesoderm induction and gastrulation in *Drosophila* (Simpson 1983; Thisse et al. 1988; Leptin et al. 1992). *Drosophila* Twist is expressed on the ventral side of the blastoderm and activates genes that are required for mesoderm development (Leptin 1991; Baylies and Bate 1996; Castanon and Baylies 2002). In the *Drosophila* embryo, a subset of myoblasts, the so-called adult muscle progenitor cells (AMPs) continues to express Twist whereas differentiating larval muscles lose Twist expression (Currie and Bate 1991; Bate et al. 1991; Cripps and Olsen

1998; Castanon and Baylies 2002). During metamorphosis, the AMPs adopt their differentiation fate and contribute to the formation of adult muscles that lose Twist expression during further myogenesis (Fernandes et al. 1991).

A *twist* ortholog is also essential for the specification and invagination of the mesoderm in the short germ beetle *Tribolium* where it is additionally expressed in a posterior growth zone during the developing germ band (Sommer and Tautz 1994; Handel et al. 2005). In the nematode *Caenorhabditis elegans*, *twist* activity revealed by reporter fusions and antibody staining was not observed during early embryonic development. However, a *twist* reporter was highly active in the postembryonic single blast cell (M) and all undifferentiated descendants of M that lose *twist* activity upon differentiation into body wall and sex muscles (Harfe et al. 1998). Recently, the existence of two potential *twist* paralogs (*CapI-twi1* and *CapI-twi2*) was reported in the polychaete *Capitella* sp. I. *CapI-twi1* is a zygotic gene expressed during larval development within the mid-body mesoderm, in cells of the head mesoderm, in the stomodaeum, in regions of the developing foregut, as well as in the hindgut. *CapI-twi2* shows a broad expression in the developing mesoderm where it partially overlaps with *CapI-twi1* (Dill et al. 2007).

In contrast to invertebrate development, Twist is not involved in chick and mouse gastrulation (Wolf et al. 1991; Tavares et al. 2001; Technau and Scholz 2003). Moreover, while high Twist levels promote somatic myogenesis in the *Drosophila* embryo (Baylies and Bate 1996), Twist is a negative regulator of vertebrate myogenesis that blocks the activity of myogenic bHLH and MEF2 members and is therefore excluded from the developing myotome (Spicer et al. 1996; Technau and Scholz 2003). These results indicate significant differences in Twist function between vertebrates and insects (Technau and Scholz 2003). Controversially, Anant et al. (1998) reported that the persistent expression of *twist* inhibits the differentiation of indirect but not direct flight muscles in *Drosophila*. These results indicate that Twist functions may not only differ between taxa but also within a single organism, depending on developmental- and tissue-specific contexts. The identification of *twist* orthologs from further species and the analysis of their spatiotemporal expression during development could therefore elucidate our understanding of *twist* gene function.

In this study, we report the identification of a *twist* ortholog (referred to as *Pdu-twist*) in the marine annelid *Platynereis dumerilii* and utilize *Pdu-twist* gene expression to follow mesoderm formation during larval development, growth up to adulthood, and regeneration of the posterior body segments. Our findings imply differences between larval mesoderm development and the formation of mesodermal tissues from a posterior growth zone during adulthood and regeneration revealing alternative segmentation modes during the animal's life cycle.

Material and methods

Platynereis dumerilii culture

Standard *Platynereis dumerilii* culture methods were followed (Hauenschild and Fischer 1969). Regenerates were produced by amputation of posterior ends from atoke specimens (for a detailed protocol, see Pfeifer et al. 2012).

Cloning of partial and full-length *Pdu-twist* cDNAs

We isolated a fragment of *Pdu-twist* by semi-nested PCR after reverse transcription of mRNA derived from 48 h embryos with the Omniscript RT Kit (Qiagen). The following degenerate primers for *Pdu-twist* were used: forward GCC AAC GTC CGC GA(AG) (AC)G(AGCT) CA(AG) (AC)G, nested forward CCT TCG CCC CGC TG(AC) G(AGCT)A A(AG)A T(ACT)(AG) T, and reverse TCC ATG CGC CAC ACG (GC)(AT)(AG) AA(AGCT) GC(AG)TA. A PCR product of the expected size was gel purified using QIAquick Gel Extraction Kit (Qiagen) subcloned into pCRII-TOPO vector (Invitrogen) and sequenced (Seqlab).

Full-length cDNA clones were obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) on an embryonic 48-h cDNA library using sequence-specific primers for *Pdu-twist* (forward ACG TGC GCG AGC GAC AGA GTA CAC and reverse GCC ACG GTA ACT GCA GGA CGG GGG AA) and the primers supplied with the GeneRacer-Kit (Invitrogen). The resulting fragments were gel purified using QIAquick Gel Extraction Kit (Qiagen), subcloned into pCRII-TOPO vector (Invitrogen), and sequenced (Seqlab).

The complete cDNA sequence of *Pdu-twist* will be submitted to the NCBI (Acc. Nr. KF241717) nucleotide sequence database.

Gene orthology assessment

Protein sequences of a selected number of species were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov) and aligned using CLUSTALX (Thompson et al. 1997). This alignment spanning the conserved bHLH domain was used to calculate a 1,000-fold bootstrapped phylogenetic tree using the Neighbor-Joining method (Saitou and Nei 1987), excluding all positions with gaps in the alignment and correcting for multiple substitutions, using the program Tree-puzzle 5.2 (Schmidt et al. 2002).

Preparation and fixation of larvae and animal tissues

Larvae, regenerates, and posterior ends were fixed overnight in 3.7 % formaldehyde in phosphate-buffered saline (PBS, pH 9.5) at 4 °C. After several washes in PBS containing 0.1 % Tween-20 (PTw), specimens were rinsed twice with methanol and stored at −25 °C for later in situ hybridization. After

rehydration in a descending series of 5-min methanol/PTw washes, regenerates and posterior ends were digested for 10 min with 10 μg/ml proteinase K in PTw, while larvae were digested 5 min. Digestion was stopped by washing the samples with 2 mg/ml glycine in PTw for 2×5 min.

In situ hybridization

In situ hybridization was performed according to Pfeifer et al. (2012). For probe synthesis, the 632-bp 5′ RACE fragment of *Pdu-twist*, encoding the entire bHLH-domain, was subcloned into the pCRII-TOPO vector (Invitrogen). Probes were generated using the Sp6- and T7-Megascript Kits (Ambion/Lifescience) and applied to the samples in a final working concentration of 2 ng/μl. Before mounting in Euparal (Carl Roth), samples were dehydrated in an increasing ethanol/water (*v/v*) series (30, 50, 70, 80, 90, 96, 98, and 100 %).

Results

Cloning of a *Platynereis twist* ortholog

A *Platynereis twist* fragment of 243-bp length spanning the bHLH domain was isolated by RT-PCR with degenerated primers derived from conserved regions in the bHLH domain. The fragment was used to design sequence-specific primers for 5′ and 3′ RACE. Due to RLM-RACE PCR, we isolated a fragment of 632-bp length in the 5′ RACE reaction and a fragment of 1,292-bp length in the 3′ RACE reaction. With the sequence information obtained from the RACE, we built a contiguous nucleotide sequence of 1,895 bp representing the complete cDNA of *Pdu-twist* and, as proven by PCR on genomic DNA (data not shown), also the complete genomic sequence of *Pdu-twist*. Analysis of this sequence revealed a potential open reading frame of 666 bp, followed by a 1.1-kb-long 3′-UTR which is often found in genes encoding the bHLH domain (Spring et al. 2000). The open reading frame encodes a polypeptide of 222 amino acids, which shares a high similarity to other known members of the Twist family (Fig. 1). The deduced sequence of *Platynereis* Twist contains a basic DNA binding and dimerization motif common to bHLH proteins and a C-terminal motif of 14 amino acid residues (WR motif) characteristic for most members of the Twist subfamily (Fig. 1), but absent from all less related bHLH proteins (Spring et al. 2000).

Platynereis Twist (Pdu-Twist) clusters with other members of the Twist-like family of transcription factors

The analysis of the aligned sequences (Fig. 1b) revealed that the Pdu-Twist bHLH domain shows the highest score of similarity to the lophotrochozoan (90–94 %) and vertebrate (89–90 %) members of the Twist family. The Twist ortholog of the ecdysozoan *Caenorhabditis elegans* shares only a 62 % sequence identity which is just slightly higher than the score for mouse Scleraxis (59 %). However, inside, the Twist cluster Pdu-Twist groups together with Hr-Twist (Soto et al. 1997) from another unequal spiral cleaving annelid. The sequence of the WR motif (ERLSAYSVRMEG) is equivalent to the sequence found in almost all other Twist orthologs (Fig. 1b). Phylogenetic analysis was performed with amino acid sequences from the bHLH region of Pdu-Twist, known members of the Twist family and other bHLH factors using the Neighbor-Joining algorithm. In the resulting tree (Fig. 1c), Pdu-Twist forms a cluster with the proteins encoded by twist and its orthologs. Other proteins containing a bHLH domain formed distinct groups that are clearly separated from the Twist cluster. We therefore suggest that *Platynereis* Twist is a real member of the Twist family of bHLH transcription factors.

Pdu-twist expression during larval development

To examine the expression pattern of *Pdu-twist*, we performed in situ hybridization using a ribo-probe generated from a 632-bp 5′ RACE fragment of *Pdu-twist*. The expression of the *Pdu-twist* mRNA was examined from 24 h post-fertilization (hpf) up to 120 hpf (Fig. 2). Analysis with differential interference contrast microscopy reveals that *Pdu-twist* expressing cells are located directly between ectodermal and endodermal cells in the 24 hpf larva (Fig. 2 (a, a′, b)). At this time, expression is detectable in three bilateral domains along the anterior–posterior axis (Fig. 2 (a, a′, b)) while another expression domain can be observed in the position of the presumptive stomodaeum (Fig. 2 (a, b, arrowheads)). In addition, two small *Pdu-twist* expressing patches can be observed on both sides of the 24 hpf larva (Fig. 2 (a, a′, arrows)). According to their location in the episphere, it is most convincing that these cells refer to the transitory larval head kidneys (Hasse et al. 2010). During further development, *Pdu-twist*-positive cells of the three bilateral domains expand around the ventral and dorsal chaetal sacs and give rise to the trunk musculature (Fig. 2 (c–i)), while the stomodeal expression increases in intensity and labels the developing pharyngeal muscles (Fig. 2 (c, e, arrowheads, i, g)). During nectochaete stages, *Pdu-twist* expression of the trunk musculature is slightly decreased (Fig. 2 (i–j′)) compared to earlier stages whereas a robust expression can be observed within the pharyngeal musculature including the pharyngeal retractors (Fig. 2 (i, arrows)). Expression within the larval head kidneys is no longer detectable at this stage. We also observed *Pdu-twist* expression in two cells between the pygidium and the last larval segment 120 hpf (Fig. 2 (j′, red arrow)).
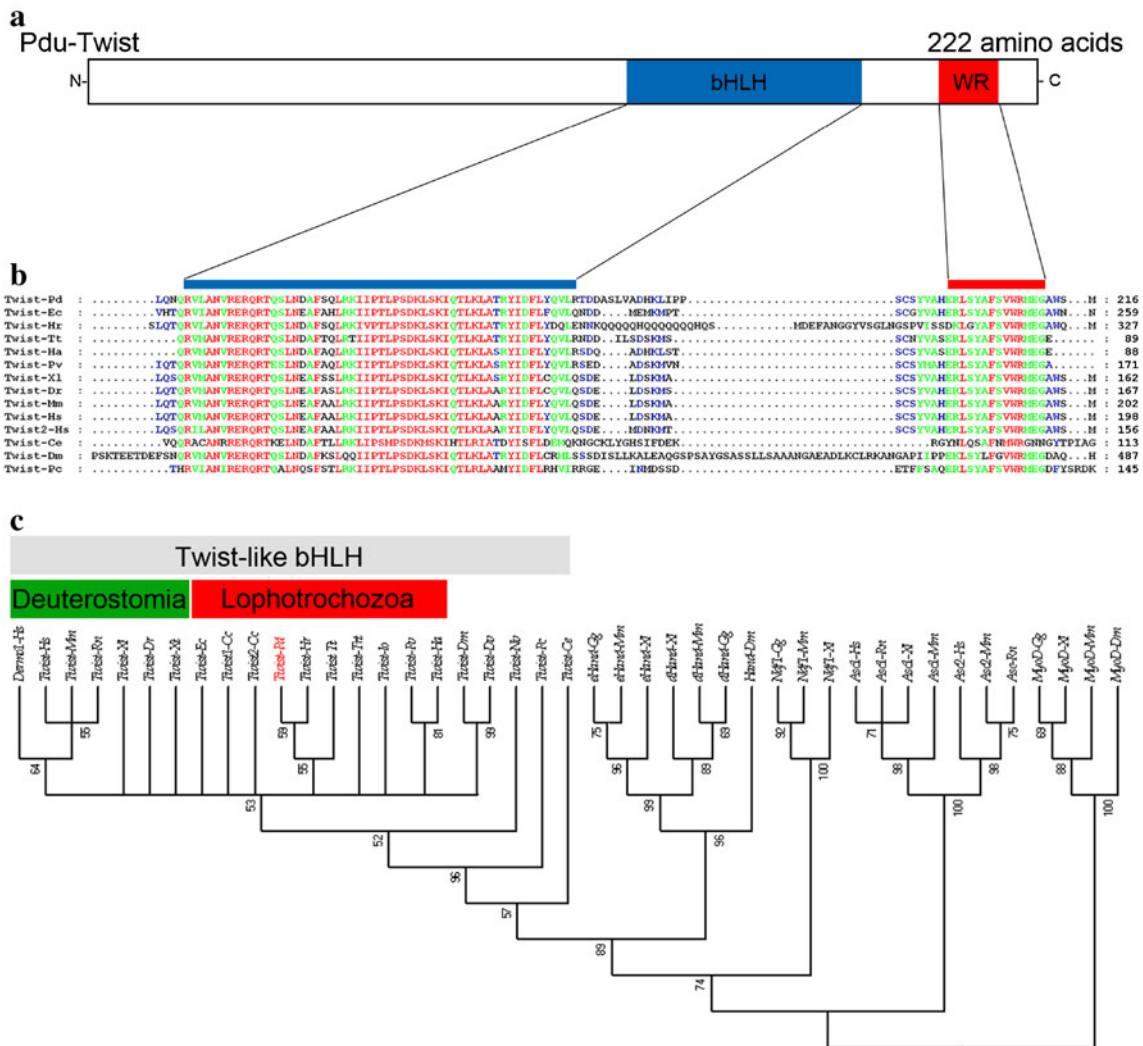
Fig. 1 Phylogenetic analysis of *Platynereis dumerilii* Twist. **a** Proposed domain architecture of Pdu-Twist. **b** Sequence alignment of *Platynereis* Twist with Twist proteins from other species and Twist2-Hs (Dermo1-Hs) in the regions of the bHLH domain and the WR motif. Members of the Lophotrochozoa and vertebrates show the highest degree of conservation. **c** Phylogenetic tree of Twist-like bHLH and related transcription factors reconstructed with the Neighbor-Joining method. Sequences were taken from the NCBI database. Bootstrap values result from 1,000 replicates. All branches with a bootstrap value <50 % were collapsed. Pdu-Twist clusters with other known Twist homologs and this cluster is clearly separated from the clusters built by other bHLH domain containing proteins. Abbreviations: *Pd*, *Platynereis dumerilii*; *Ec*, *Enchytraeus coronatus*; *Hr*, *Helobdella robusta*; *Tt*, *Transenella tantilla*; *Ha*, *Helix aspers*; *Pv*, *Patella vulgata*; *Xl*, *Xenopus laevis*; *Dm*, *Drosophila melanogaster*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *Ce*, *Caenorhabditis elegans*; *Pc*, *Podocoryne carnea*

Pdu-twist expression during growth, i.e., posterior addition of segments

Our analysis revealed *Pdu-twist* mRNA-positive cells within the simultaneously forming segments of developing larvae. In contrast to larval segment formation, new segments are consecutively formed by a posterior growth zone up to adulthood (Fischer et al. 2010). Hence, we were interested in whether the mesodermal marker gene *Pdu-twist* is also expressed in the posterior segmentation zone of adult worms (Fig. 3). *Pdu-twist* mRNA expression is detected in the posterior growth zone but also in iterated stripes in the youngest body parts suggesting first signs of segmentation (Fig. 3b–d). Within the posterior growth zone, *Pdu-twist* expression is detectable in lateral domains and some dorsal extensions, which might indicate the presence of defined mesodermal anlagen from which cells contribute to the formation of the new segments. No expression is detected along the ventral side (Fig. 3a) but *Pdu-twist* is expressed in cells within the lateral forming parapodia (Fig. 3a, f). In older segments, *Pdu-twist*-positive cells are restricted to the parapodial base, whereas in younger segments, *Pdu-twist* expression extents dorsally (Fig. 3d, f). In fully differentiated segments, we detected only faint *Pdu-twist* expression in cells located at the anterior parapodial base (data not shown).

Fig. 2 In situ hybridization against *Pdu-twist* mRNA in different larval stages of *Platynereis dumerilii*. *Asterisks* indicate the stomodaeum. *a, a′ Pdu-twist* expression in different focal planes of a 24-hpf larva. *Arrows* mark *Pdu-twist* expression domains that flank the stomodaeum. *Pdu-twist* expression in the developing stomodaeum is highlighted by *arrowheads*. Anterior is *up*, posterior is *down*. *b* Posterior view of a 24-hpf larva. *Pdu-twist* expression in the developing stomodaeum is highlighted by *arrowheads*. Ventral is *down*, dorsal is *up*. *c, c′ Pdu-twist* expression at 36 hpf. *Arrowheads* point to *Pdu-twist* expression in two bilateral domains around the stomodaeum. Two different focal planes are shown, anterior is *up* and posterior is *down*. *d* Posterior view of a 36-hpf larva. Dorsal is *up*, ventral is *down*. *e, e′ Pdu-twist* expression in different focal planes of a 48-hpf larva. *Pdu-twist* expression ambilateral of the stomodaeum is marked by *arrowheads*. Anterior is *up*, posterior is *down*. *f* Lateral *Pdu-twist* expression around the chaetal sacks of a 48-hpf larva. *g, g′ Pdu-twist* expression at 58 hpf in two different focal planes. Anterior is *up* and posterior is *down*. *h* Posterior *Pdu-twist* expression at 58 hpf. *i, i′ Pdu-twist* expression in different focal planes of a 72-hpf larva. *Arrows* indicate *Pdu-twist* expression in the pharyngeal retractor muscles. Anterior is to the *left*, posterior is to the *right*. *j, j′* Different focal planes of the *Pdu-twist* expression in a 120-hpf larva. The *red arrow* marks *Pdu-twist* expression in two cells within the pygidium. Anterior is to the *left*, posterior is to the *right*. *Scale bars* 50 μm
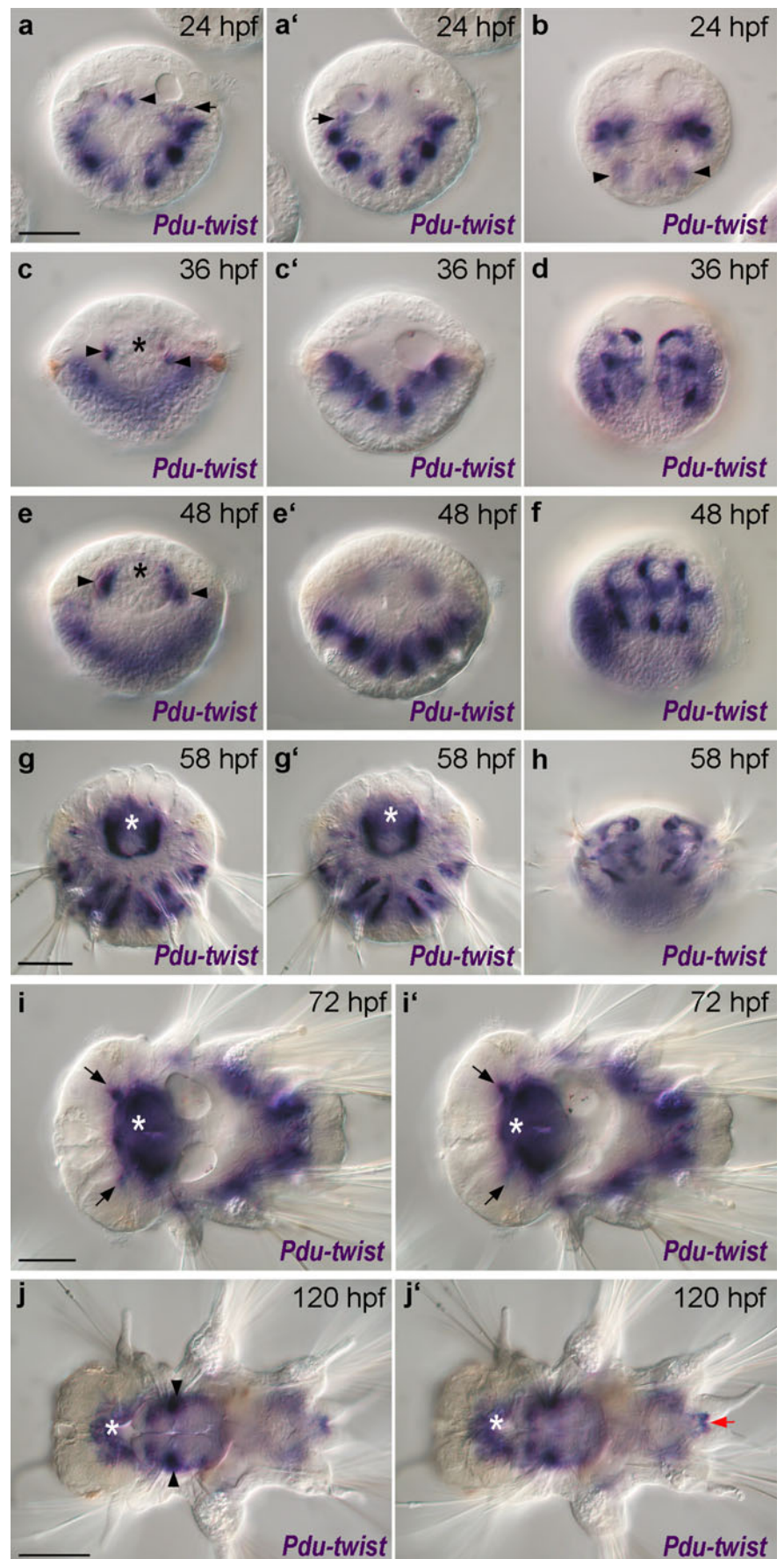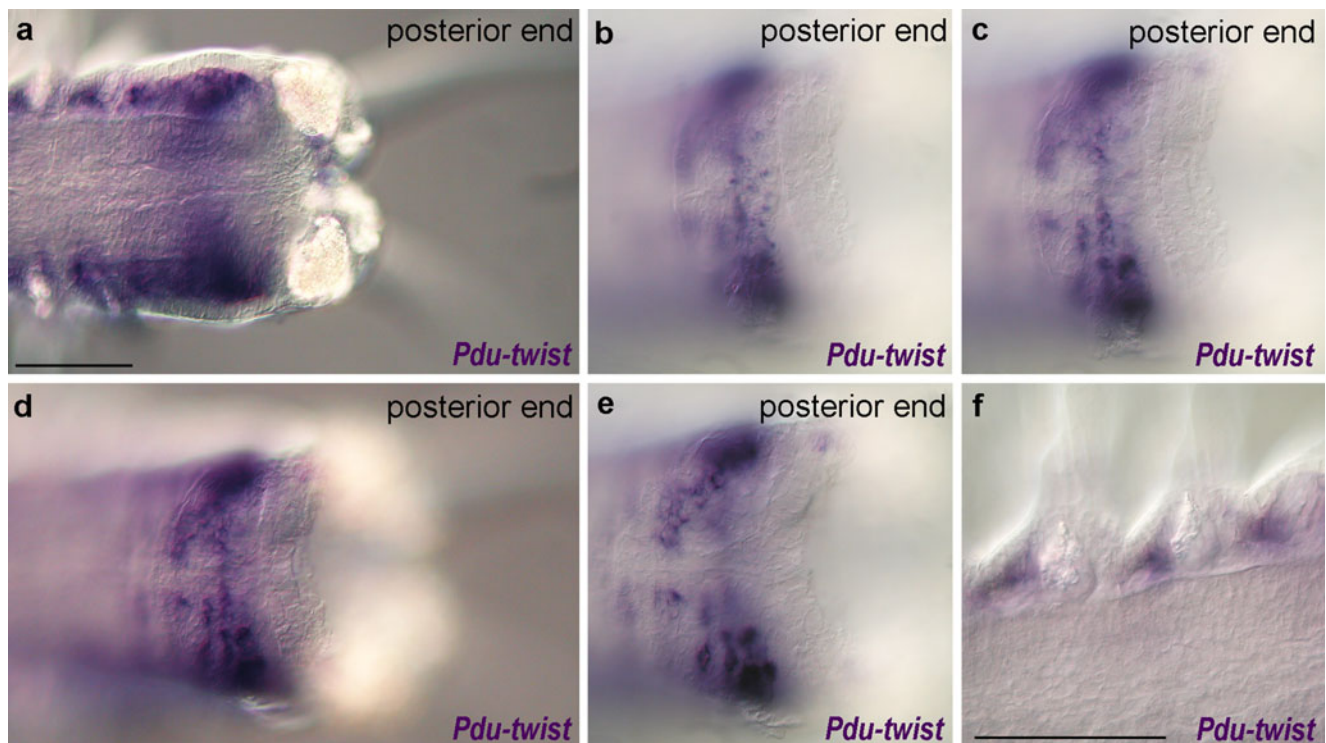
Fig. 3 *Pdu-twist* expression in posterior ends of *Platynereis dumerilii*. Posterior is to the *right* in all panels. **a–f** In situ hybridization against *Pdu-twist* mRNA in posterior ends. **a** *Pdu-twist* expression in a posterior end from a ventral view. **d** Dorsal view of a posterior end showing *Pdu-twist* expression. **b, c, e** Close-up of a posterior end with *Pdu-twist* expression from a dorsal view. **f** Close up of a. *Pdu-twist* expression in the anterior part of the parapodia in differentiated segments of the posterior end. *Scale bars* 100 μm

Taken together, these results indicate that the mesodermal tissues in each newly formed segment arise from defined anlagen within the posterior growth zone that are characterized by *Pdu-twist* expression.

Pdu-twist expression during different stages of caudal regeneration

A characteristic feature of *Platynereis dumerilii* is the ability to regenerate caudal segments after the posterior end has been amputated from the adult worm (Hauenschild and Fischer 1969). Since *Pdu-twist* mRNA is expressed in distinct domains within the posterior growth zone, we wondered how the cascade of events that leads to the establishment of mesodermal tissues within each segment in normogenesis is recovered after a removal of the growth zone. Therefore, we employed in situ hybridization against *Pdu-twist* as a marker for mesoderm development and followed its expression during the regeneration periods of 1, 2, 3, 4, 5, and 8 days post-amputation (dpa).

Already 1 day after wounding, *Pdu-twist* expression is detectable below an epithelial cell layer posterior to the amputation site (Fig. 4 (a, a′)). Two discrete expression domains are located laterally while one domain appears on the dorsal side of the regenerate (Fig. 4 (a, a′, arrowheads)).

Two days post-amputation, the three expression domains have extended to a horseshoe-like structure leaving a ventral opening within the formed blastema (Fig. 4 (b, b′)). Compared to 1 dpa, the expression has increased in intensity. Three days post-amputation, the budding of the anal cirri can be observed at the posterior end of the regenerate (Fig. 4 (c, c′); de Rosa et al. 2005). *Pdu-twist* is expressed at the base of the anal

Fig. 4 *Pdu-twist* expression during various stages of caudal regeneration ▶ in *Platynereis dumerilii*. Anterior is to the *left* and posterior is to the *right* in all panels. The *red line* marks the side of amputation. The regenerate is on the *right side of the red line*, the original segment is on the *left* side. *a* *Arrowheads* point to three *Pdu-twist* expression domains 1 dpa. Dorsal view. *a′* Close-up of a. *b* *Pdu-twist* expression 2 dpa. Ventral view. *b′* Close-up of *b*. *c* Dorsal *Pdu-twist* expression 3 dpa. *Green arrowhead* points to the circular expression domain around the anus. *c′* Ventral *Pdu-twist* expression 3 dpa. *Arrow* marks *Pdu-twist* mRNA-positive cells in the region of the new established posterior growth zone. *Arrowhead* points to *Pdu-twist* expression on the base of the anal cirri buds. *c″* and *c‴* Close-up of *c′*. *d* *Pdu-twist* expression 4 dpa. *e* Five days post-amputation, the *Pdu-twist* expression is detected in a segmental iterated stripe pattern along the anterior posterior axis of the regenerate. Expression within the posterior growth zone is marked by a *red arrowhead*. *e′* *Pdu-twist* expression in stripes is highlighted by *arrows*. *Green arrowhead* points to *Pdu-twist* expression around the anus. *f, f′* *Pdu-twist* expression 8 dpa in different focal planes. *g* *Pdu-twist* is expressed within the newly developed parapodia. *h* Regenerate 8 dpa from a dorsal view. *Red arrowhead* marks *Pdu-twist* expression in the posterior growth zone. The *green arrowhead* points to the *Pdu-twist* expression around the newly formed anus. *Scale bars* 100 μm
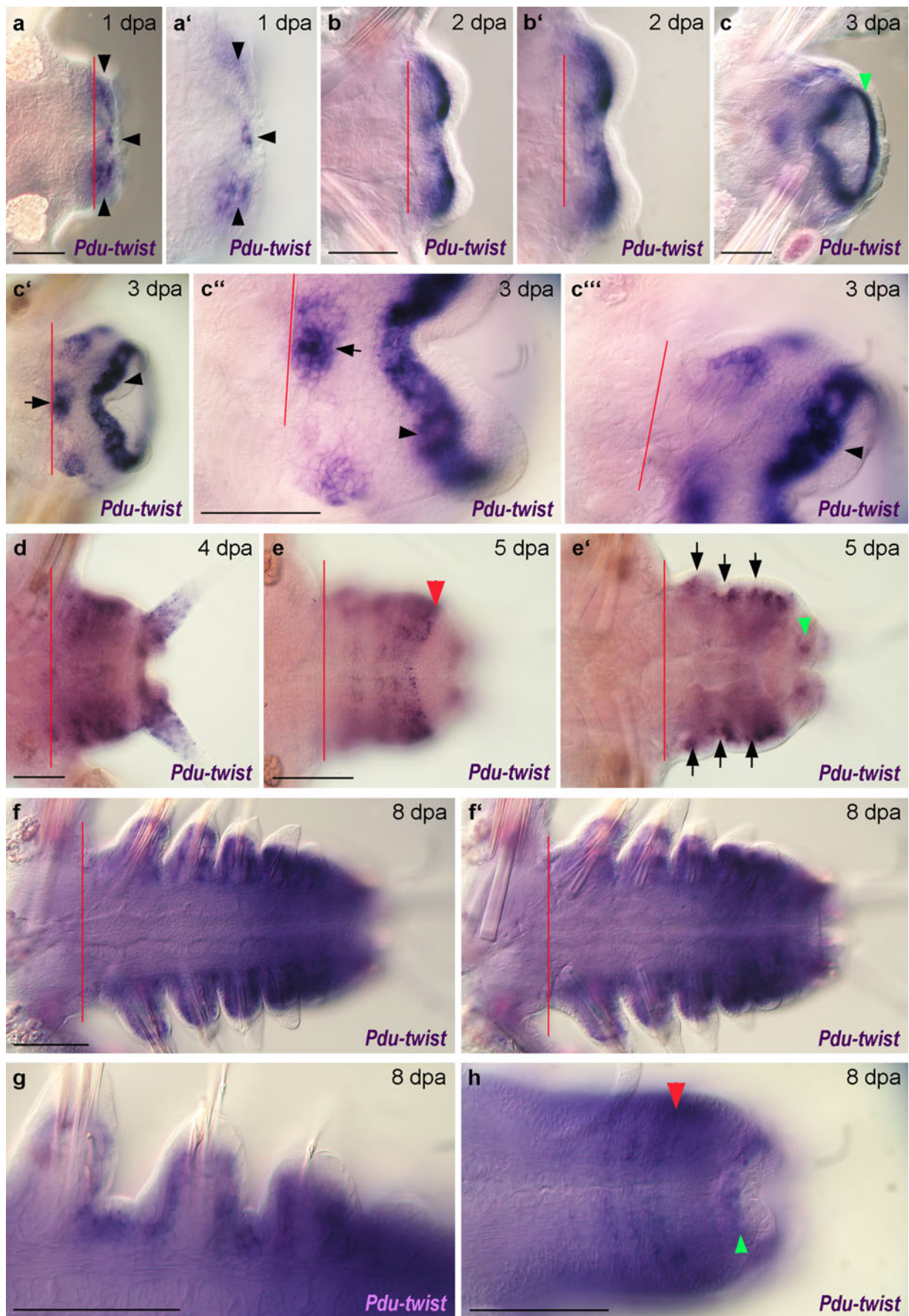
cirri buds (Fig. 4 (c′-c‴, arrowheads)) and around the anus opening, which probably refers to the prospective sphincter muscle cells (Fig. 4 (c, green arrowhead)). In addition to the horseshoe-like expression domain already observed 2 dpa, a ventral expression patch is now detectable (Fig. 4 (c′, c″, arrow)). Four days post-amputation, the beginning of the segmentation process is revealed by stripes of *Pdu-twist*-positive cells in the center of the prospective segments while expression is still detectable around the anus opening (Fig. 4 (d)). At this time, single *Pdu-twist*-positive cells are also detectable at the base and within the anal cirri (Fig. 4 (d)). After one more day, a new pygidium has formed and the segmentation of the regenerate becomes apparent by the forming parapodial buds (Fig. 4 (e′, arrows)). The circular expression of *Pdu-twist* within the pygidium suggests the formation of the anus sphincter musculature (Fig. 4 (e′, green arrowhead)). *Pdu-twist* is also expressed in iterated stripes between the pygidium and the original segment (Fig. 4 (e and e′, arrows)) while the newly established posterior growth zone is still denoted by the horseshoe-like expression domain of *Pdu-twist* at this stage (Fig. 4 (e, red arrowhead)). Eight days post-amputation, up to seven segments have formed that vary in their differentiation status. While the oldest segments already bear parapodia, only lateral buds are present in the middle segments and the youngest segments show no morphological signs of parapodia formation. Interestingly, while lateral stripes of *Pdu-twist*-positive cells are detectable in the youngest segments (Fig. 4 (h)), *Pdu-twist* expression intensity decreases during parapodia development (Fig. 4 (f–g)). *Pdu-twist* is still expressed in the posterior subterminal proliferation zone in a horseshoe-like domain and within the sphincter musculature (Fig. 4 (h)). Taken together, our results indicate that *Pdu-twist* might be involved in the reestablishment of mesodermal tissues within the blastema and the subsequent formation of new segments after caudal amputation.

Discussion

A *twist* ortholog is expressed during *Platynereis dumerilii* mesoderm development

Previous studies among a variety of bilaterian species revealed that the bHLH transcription factor Twist is essential for mesoderm development (Technau and Scholz 2003). In accordance, expression of *twist* within cells of mesodermal origin has been described in several lophotrochozoans (Nederbragt et al. 2002; Dill et al. 2007). In this work, we identified a potential *twist* ortholog in the annelid *Platynereis dumerilii*. Sequence analysis of *Platynereis dumerilii twist* (*Pdu-twist*) unveils the presence of the highly conserved bHLH domain and the C-terminal WR motif known from

other orthologs like *Drosophila* Twist, the founding member of the Twist-like subfamily. A phylogenetic analysis based on amino acid identity in the bHLH domain sequences of the Twist homologs revealed between 59 and 85 % conserved residues in comparison to *Drosophila* Twist, whereas the bHLH domains show a higher degree of conservation within the vertebrates (Castanon and Baylies 2002). In the Neighbor-Joining tree derived from the phylogenetic analysis of the bHLH domains, the family of the Twist-like transcription factors shows a distinct systematic separation to the other families of bHLH domain containing transcription factors. The phylogenetic branch of the Twist homologs exhibits a pronounced polytomy clustering of the chordate members, the Lophotrochozoa, Ecdysozoa, and the cnidarian phyla. Only the Twist homolog of *Caenorhabditis elegans* displays an atypical position at the root of the phylogenetic tree, probably representing a long-branch attraction artefact of the Neighbor-Joining analysis. *Platynereis twist* can be found in the "Lophotrochozoan cluster" and in systematic neighborhood to the Twist homologs from *Tubifex tubifex* and *Helobdella robusta*, demonstrating that *Pdu-twist* represents a real member of the Twist-like family of transcription factors.

Former studies on *Helobdella robusta* and *Patella vulgata* (Soto et al. 1997; Nederbragt et al. 2002) reported the identification of only one *twist* ortholog, which would be in accordance to our findings in *Platynereis*. However, in the polychaete *Capitella* sp. I, the presence of two *twist* paralogs has been reported indicating differential numbers of *twist* genes within these closely related taxa (Dill et al. 2007). Since variations in the number of *twist* paralogs is common, even among vertebrate species (Germanguz et al. 2007), the number of *twist* genes in the genome might not be a valid criterion for understanding phylogenetic relationships. There are several known functions of *twist* in Bilateria. In the *Drosophila* embryo, Twist is required for the normal development of the larval musculature, whereas in vertebrates, Twist is supposed to inhibit myogenesis (Hebrok et al. 1994; Cripps and Olsen 1998; Technau and Scholz 2003). Whole mount in situ hybridization of *Pdu-twist* demonstrates that *twist* mRNA is specifically expressed in mesodermal cell packages during early larval development and within the musculature of larvae and young worms, indicating a similar function as presumed for other invertebrate embryos and larvae (Michelson 1996; Technau and Scholz 2003; Dill et al. 2007; Martín-Durán et al. 2010).

Pdu-twist expression in posterior ends during adulthood and caudal regeneration

Adult worms continuously add new segments from a subterminal posterior growth zone. Within this particular region, *Pdu-twist* is expressed in lateral domains with dorsal extensions,

revealing a defined source for the mesoderm during posterior growth. Interestingly, while *Pdu-twist* mRNA is highly present in the youngest segments of adult worms, its expression decreases during further development and is absent within mature muscles and fully differentiated segments.

We also analyzed that the establishment of mesodermal tissues is recovered after removal of this posterior growth zone. According to the results presented here, not all cells joining in blastema formation are *Pdu-twist* mRNA positive although *twist* expression is already detectable in defined domains at 1 dpa. One explanation for such an early patterning might be that, in consequence of a trauma, de-differentiating cells descend from different tissues along the amputation site and contribute to blastema formation. Thus, the assumption that the blastema in *Platynereis dumerilii* consists of cells derived from already differentiated tissues that hold only restricted potentials for re-differentiation (Tanaka and Reddien 2011) would be more convincing than a de novo formation by adult stem cells or "neoblasts." During the regeneration process, the blastema forms a pygidium and a posterior subterminal proliferation zone. Subsequently, the formation of new segments begins. A similar process has been described in the polychaete species *Perinereis nuntia* (Niwa et al. 2013). As reported by Niwa et al. (2013), the segment regeneration mode seems to be an identical but accelerated mode of the posterior segment formation process (Niwa et al. 2013). During adult segment formation in *Platynereis*, *Pdu-twist* expressing cell rows can be detected in the dorsolateral regions of the posterior ending. Comparing segment formation during the regeneration process, we detected less *Pdu-twist* expressing cells suggesting that the regeneration of segments in *Platynereis* might be an accelerated but identical mode of the normal segmentation process.

Taken together, these results reveal that we isolated a *Platynereis twist* ortholog, called *Pdu-twist*. Its expression within the developing mesoderm and forming muscles suggests a conserved function among invertebrate species.

Conclusions

In this study, we report the identification of a *twist* ortholog in the polychaete annelid *Platynereis dumerilii* and employ *Pdu-twist* expression to follow mesoderm formation during normal development and regeneration. The analysis of *Pdu-twist* expression in mesodermal cells and developing muscles at different larval stages points towards a conserved function during mesoderm development and myogenesis as postulated for other invertebrates. The continuous proliferation of segments from a posterior subterminal proliferation zone is one of the characteristic features of the *Platynereis dumerilii* life cycle. Our examination on mesoderm specification during this posterior

segmentation process indicates the presence of defined *Pdu-twist* expressing anlagen within this posterior growth zone that could serve as a proliferative reservoir for the mesoderm during adulthood. Employing *Pdu-twist* expression as a marker, we could follow the re-establishment of the posterior subterminal proliferation zone after caudal amputation for the first time in *Platynereis*. Our results reveal that a distinct *Pdu-twist* expression pattern is already detectable within the early forming blastema. This would favor a model in which the blastema in *Platynereis* is composed of dedifferentiated cells that descend from tissues along the amputation site. Furthermore, segment regeneration could be accelerated but otherwise identical with the segment formation process observed during normal posterior growth. In contrast to simultaneous segment formation within the larva, the segment appearance in adults (independent of normal posterior growth or regeneration) follows a successive program.

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