Involvement of the *Notch*-signaling pathway in the development of the two polychaete annelids *Capitella* sp. I and *Platynereis dumerilii*

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Katrin Thamm

aus Hildburghausen

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Dekan: Prof. Dr. Peter R. Schreiner
Erster Gutachter: Prof. Dr. Adriaan Dorresteijn
Zweiter Gutachter: Prof. Elaine C. Seaver, PhD

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1. Introduction

1.1 Segmentation

The existence and basal characteristics of a hypothetical common bilaterian ancestor has always led to discussions in the history of evolutionary and developmental biology (Balavoine and Adoutte 2003; De Robertis 1997; Kimmel 1996). When talking about segmentation, the first hurdle is the definition. Several authors interpret a segment as a sequentially iterated body unit repeating in an anterior to posterior progression comprising a set of characters which includes muscles, ganglia, septa, excretory organs, coelomic cavities and appendages (Minelli and Fusco 2004; Seaver 2003; Tautz 2004). Three major groups of animals show segmentation *in sensu stricto*: arthopods, annelids and vertebrates, and there are three different proposed scenarios to explain the evolution of segmentation (Davis and Patel 1999). In the early proposed phylogenies of Metazoans, segmentation was considered as a synapomorphic characteristic of all three phyla derived from a common segmented ancestor (Balavoine and Adoutte 2003; Cuvier 1817; Sedgwick 1884). Due to the protostome-deuterostome distinction at the beginning of the 20th century, this hypothesis was abolished (Grobben 1908). Vertebrate somitogenesis and segmentation in arthopods and annelids were considered to have two different origins in the evolution of bilaterians. The latter two groups were included in the group Articulata with a common segmented ancestor (Scholtz 2002). However, recent molecular data even suggest that annelids and arthropods are more closely related to unsegmented groups like molluscs (annelids) and nematodes (arthropods) respectively than to each other (Aguinaldo et al. 1997; de Rosa et al. 1999). Taking these hypotheses in account, we have to assume three possible scenarios concerning the evolution of segmentation (Davis and Patel 1999). First, it is possible that the common ancestor was segmented but segmentation was independently lost in all unsegmented phyla existing today (Patel 2003) (Fig. 1A). Even though this seems to be the hardest theory to prove right now with the accepted phylogeny of Metazoans, there are recent molecular results supporting this hypothesis (see below and 1.5). Secondly, it is possible that in the line of the protostomes a common segmented ancestor existed leading to the segmented groups of annelids and arthropods, but segmentation was lost in the related unsegmented taxa (Fig. 1B). This would be closely related to the Articulata hypothesis. Many authors support this possibility, especially due

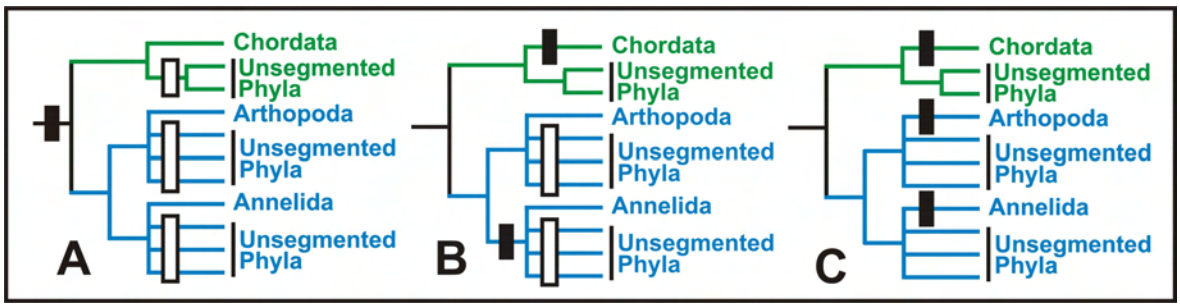


Fig. 1. Three hypotheses for the evolution of segmentation. A. A common segmented ancestor for all three groups with multiple loss of segmentation among the unsegmented phyla. **B.** Homology of segments among annelids and arthopods with loss of segmentation among the unsegmented phyla. Segmentation in chordates arose independently. **C.** Segmentation arose independently in all three phyla. Green labels Deuterostomia; blue labels Protostomia. Solid black bocks = acquisition of segmentation; solid white block = loss of segmentation (modified after Davis and Patel 1999).

to complexity of similarities of segments across arthopods and annelids (Scholtz 2002). The third theory would be the convergent evolution of segments in all three phyla, arthropods, annelids and vertebrates (Fig. 1C). The fact that segmentation in arthropods is mainly the property of the ectoderm and in vertebrates the property of the mesoderm would also account for an independent evolution of segments (Patel 2003) as well as many differences in the molecular formation of segments across the three phyla (Davis and Patel 1999; Davis and Patel 2003; De Robertis 1997; Peel and Akam 2003).

All three phyla of segmented animals have characteristic segmentation modes. Formation of segments in insects can be divided in to long-germ like patterns such as in *Drosophila* and short-germ like patterns e.g. in *Schistocerca.* Segments of *Drosophila* are being generated as early as the syncytial blastoderm where segmentation genes can act in an environment only partially confined by cell membranes, and the primordia of segments is set up almost simultaneously. The head segments of *Schistocerca* are also formed in an initial syncytium but all remaining segments are formed progressively from a posterior growth zone, thus comparable to the way of opisthosoma segmentation in the spider *Cupiennius salei* (Bentley et al. 1979; St Johnston and Nusslein-Volhard 1992).

Annelid segment formation is often compared with the leech mode of segmentation featuring teloblastic growth (Seaver 2003; Tautz 2004). Teloblasts possess stem cell character and are usually located at the posterior end of the body. They are relatively large in size, divide asymmetrically and form segmental founder cells (Seaver and Shankland 2001; Weisblat et al. 1988; Weisblat and Shankland 1985). In contrast to Oligochaetes and Hirudinea (Clitellates), no teloblasts have been found in polychaetes. Thus, the proposed ancestral mode of segmentation in annelids always leads to discussions (de Rosa et al.

2005; Scholtz 2002). In general, it can be assumed that teloblastic segmentation, extensively studied in leech, is not the basal process of segment addition in annelids since it was never found in polychaetes. In addition, it is the result of a highly derived cleavage pattern of the embryonic D-Quadrant, which is exclusively encountered in clitellates. Therefore, the polychaetes must represent the more basal group of the annelids since they possess more ancestral features in development and body plan than do the clitellates. We can also assume the ancestry of posterior addition of segments in annelids, called anamery. The existence of teloblasts in some crustacean groups can also be regarded as a derived mode of segmentation and does not represent the ancestral mode of segmentation in crustaceans, since several lower crustacean groups have a posterior growth zone and show anamery (Scholtz 2002; Tautz 2004).

Vertebrates form metameric structures called somites along the anterior-posterior axis, which give rise to the future vertebrae, ribs, intervertebral disks, skeletal muscles and a large part of the dermis of the skin (Freitas et al. 2005). Somites are paraxial mesodermal structures, i.e. located bilateral to the axial midline of the embryo. They are formed in regular intervals from the unsegmented presomitic mesoderm. The number of somites and also the cycling of somite formation is specific for each organism, and has been extensively studied in zebrafish, mouse and chicken (Rida et al. 2004). The paraxial mesoderm in which the somites are generated results from cell migrations within the primitive streak, basically immigration and extension of cell conglomerates.

The question of ‘homology’ or ’convergence’ of segmentation across the animal kingdom has again become a serious debate in the last decade on the basis of molecular studies. The experiments comparing various genes involved in the segmentation of arthopods, annelids and/or vertebrates has contributed to that debate (Aulehla and Herrmann 2004; Bessho and Kageyama 2003; Dale and Pourquie 2000; Fusco 2005; Gossler and Hrabe de Angelis 1998; Pourquie 2003; Seaver 2003; Stern and Vasiliauskas 2000; Tautz 2004). The authors of these studies want to find out the basal state of the molecular network involved in the formation of segments in one phylum and they wish to determine the possible common signaling pathways responsible for segmentation in two of the three or even all segmented groups.

One intensively studied example includes the segment polarity gene *engrailed*, which marks the posterior border of a future segment in *Drosophila* and is part of the segmentation network of gap-genes, pair-rule genes, segment polarity genes and hox-genes

(Patel 1994; Tautz and Sommer 1995). *Engrailed* is expressed in the anterior part of a parasegment representing the initial metameric units in *Drosophila*. Later on, the posterior part of a parasegment and the following anterior part of the next segment translate into the formation of the final segment in the *Drosophila* embryo (Martinez-Arias and Lawrence 1985). This parasegmental organization and the conservation of *engrailed* expression was examined and proven in all four major groups of arthropods including insects, crustaceans (Patel et al. 1989), myriapods (Hughes and Kaufman 2002) and chelicerates (Damen 2002). The first *engrailed* gene in annelids examined was in the leech *Helobdella*. Expression patterns and functional studies point towards a role in neurogenesis, but not in segmentation (Lans et al. 1993; Seaver and Shankland 2001; Shain et al. 2000). The same pattern seems to emerge from expression patterns in the oligochaete *Pristina leidyi* (Bely and Wray 2001) and the polychaetes *Chaetopterus, Hydroides elegans* and *Capitella* sp. I (Seaver and Kaneshige 2006; Seaver et al. 2001). A significant exception in larval expression displays the *engrailed* gene of *Platynereis dumerilii*. The transcript of *Pdu-en* is located in the ectoderm as stripes and is supposed to mark the borders of the forming segments (Prud'homme et al. 2003). Functional experiments (like in *Helobdella*) have to be performed to get more insights into the role of *engrailed* during the segmentation process of annelids.

Engrailed was also examined in vertebrates and basal deuterostomes. In zebrafish, where it is localized anterior to the developing somites, but appears after the formation of segments. Additionally, it is only associated with a specific subset of muscle cells within the somite (Patel et al. 1989). Interestingly, the *Branchiostoma engrailed* homologue shows expression domains in border formation suggesting a role in segmentation. Functional experiments have to be performed to confirm this assumption (Holland et al. 1997). An *engrailed* gene in the basal ascidians seems to be expressed in the developing neural ectoderm, but does not show a segmentally arranged expression pattern, in contrast to the expression of the *engrailed* gene in *Branchiostoma* (Jiang and Smith 2002). Furthermore, the authors are not sure if the lack of an unsegmented mesoderm in the ascidia is a derived character of tunicates or if it represents an ancestral feature of the predicted unsegmented ancestor of vertebrates later on.

The ancestry of segmentation and its basal network can be examined from the perspective of the arthropods, which was done in the case of *engrailed*, but studies can also be performed from the perspective of vertebrates. The *Notch* signaling pathway is known to

be a major component of somitogenesis in vertebrates (see 1.5). *Notch*, its ligand *Delta* and the target genes *hairy* have been isolated and analysed in the basal arthopod *Cupiennius salei* (Stollewerk 2002). Functional results point towards common mechanisms of segmentation between chordates and arthopods (Stollewerk et al. 2003). Thus, we studied all three components in the third segmented phylum, the annelids. Two polychaetes, *Capitella* sp. I and *Platynereis dumerilii*, were used to examine the expression patterns of *Notch* and associated components of this pathway (see 1.5).

1.2 The polychaetes

1.2.1 *Capitella* sp. I

Capitella sp. I is a cosmopolitic polychaete resembling the earthworm in general appearance. *Capitella capitata* (Fabricius 1780) has been demonstrated to be a sibling species complex, which includes *Capitella* sp. I (Grassle and Grassle 1976). The sibling species differ in the structure of their eggs and ovarian follicle cells as well as in the structure of the genital spine, sperm and larval morphology (Eckelbarger and Grassle 1983; Eckelbarger and Grassle 1987; Eckelbarger and Grassle 1987). The karyotypes in the *Capitella* complex are not similar and analysis of alloenzymes also showed differences (Grassle et al. 1987; Wu et al. 1991). Detailed description of development and morphology in *Capitella capitata sensu stricto* refer to *Capitella* sp. I (Seaver et al. 2005).

Capitella sp. I lives as a burrowing worm in the sandy mud and as a scavenger feeder. The polychaete survives under very low oxygen conditions and in extremely polluted water. Its ability to survive in extreme environmental conditions makes it a useful indicator for organic polution. Ecological studies with *Capitella* sp. I as a bioindicator are often performed to test different environmental conditions like low sulfide concentrations or cadmium concentration in the water (Gamenick et al. 1998; Mendez and Baird 2002; Selck and Forbes 2004). *Capitella* sp. I and its development was first described in detail by Eisig (1890). The eggs measure up to 200µm. Embryonic and larval development occur in a brood tube, and the staging from St. 1 to St. 9 (after Seaver et al. 2005) is depicted in Fig. 2A. Up to 250 embryos grow within a single brood tube. After hatching, larvae are competent to undergo metamorphosis, which can also be induced in the lab by adding mud to St. 9 old larvae (Butman and Grassle 1992). Embryos can also be removed from the brood tubes with forceps and easily cultured in ASW (artificial sea water), where they

develop into metatrochophore larvae. After metamorphosis, it will take approximately 10 to 12 weeks until the young worms are mature enough to reproduce, which means a very short generation time in *Capitella* sp. I.

The embryonic development was described previously in detail including the staging of early cleavage embryos (Werbrock et al. 2001) (Fig. 2A). First cleavages are unequal and spiral. Gastrulation is epibolic. At St. 3 of development, the embryo elongates and forms a so called 'prototrochal girdle' without cilia, which represents the precursor cells for the prototroch. A mouth develops at late St. 3. A few hours later at St. 4, both ciliary bands, prototroch and telotroch, are visible. The lecitotrophic larvae is filled with yolk. Segmentation begins around that stage with the formation of bilateral structures called belly plates by Eisig (1890). They represent small lateral regions of cells, where the nuclei, when visualized with the nuclear stain Hoechst, are packed at higher density than in the surrounding tissue (Fig. 2B1 and 2). At St. 5, the larvae are bottom dwellers and have developed one pair of eyes. Segmentation proceeds with the expansion of the belly plates towards ventral, dorsal and posterior. The first five to six segments are visible soon after and appear almost simultanously (Fig. 2C1 and 2). The presumptive segment forming tissue expands and by St. 6, nine to ten segments have been formed. From this stage onwards, the belly plates expand towards the dorsal side (Fig. 2D1 and 2) and additional segments are now added by the posterior growth zone in front of the telotroch. The larvae have a barrel shape and a well developed neurotroch is visible between the edges of the two lateral belly plates at the ventral midline. Pronounced muscle contractions can be observed and the larvae are positive phototactic. They swim in a corkscrew like fashion in the laboratory dish. St. 7 larvae possess chaetae and grow steadily. The anterior chaetae are simple ones and the more posterior chaetae display hooded hooks (Schweigkofler et al. 1998). There is still yolk visible in the head of the larvae. Belly plates meet at the ventral midline to form the ventral nerve cord and gradually the neurotroch disappears. At St. 8 of development, regionalization along the gut is now visible and all thirteen larval segments are formed (Fig. 2E1-3). The midgut is straight from anterior to posterior and mostly green in colour. Ganglia are visible at the ventral nerve cord. The yolk is now completely absent from the head and mouth region as well as from the rest of the larvae. All segments bear well developed chaetae. Larvae are very active and swim fast through the dish. The last stage before metamorphosis, St. 9, possesses a curved midgut with convolutions. Pre-

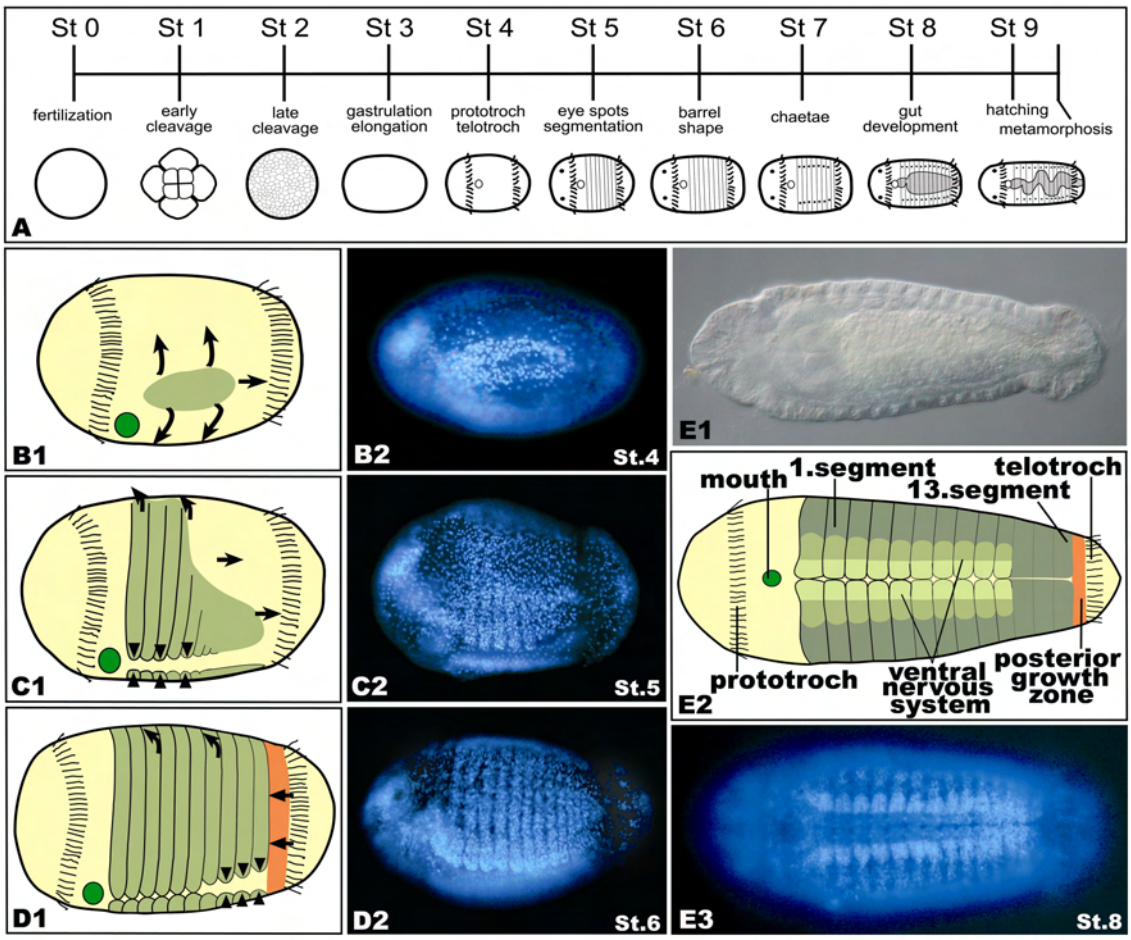


Fig. 2. Larval development of *Capitella* sp. I. A. Schematic of embryonic and larval development. **B1, 2.** St. 4; lateral presumptive segmental tissue called 'belly plates' expand in the labeled directions (arrows). **C1, 2.** St. 5; first signs of segmentation (arrowheads). Segmented part of both belly plates moves to the ventral midline (arrowheads). Dorsal and posterior expansion of the future segments (arrows). **D1, 2.** St. 6; belly plates are segmented and display the first 9-10 larval segments. The last three to four segments are added by the posterior growth zone (arrows) (orange region). **E1-3.** St. 8; thirteen segments are formed during larval development of *Capitella* sp. I. Belly plates meet at the ventral midline to form the ventral nerve cord. B1-D2 ventrolateral views. E1-3 ventral views. B1, C1, D1 and E2 display schematics of the segmentation process. B2, C2, D2 and E3 were exposed to Hoechst 33342. E1 shows a DIC image.

metamorphosing larvae swim slow and are extremely elongated. Once coming into contact with mud, they undergo metamorphosis into juvenile stages, which look like small worms. Following metamorphosis, they grow steadily by adding one segment every three days.

The previous description of a two-phased process of segmentation includes at first the belly plates as presumptive tissue for segmentation of the first ten larval segments. Then the typical annelid posterior addition of segments follows. These two scenarios and developmental sequences of segmentation in *Capitella* sp. I were examined and demonstrated by BrdU experiments (Seaver et al. 2005). It displays a special mode in contrast to the trochophora of *Platynereis dumerilii*, which forms only three larval segments in almost the same amount of time (section 1.2.2).

Capitella sp. I displays certain characteristics, which makes it an ideal polychaete to study aspects of developmental biology. 1) It has large eggs, which can easily be manipulated e.g. by injection etc. 2) The generation time of ten weeks is very short and 3) The animals reproduce throughout the entire year, so that embryos and larvae are available at any time. 4) It is a cosmopolitan and tolerant towards changing conditions, which also makes it an ideal bioindicator. 5) Feeding with mud can be done easily in the lab and does not require a high effort. 6) A number of molecular and cell biological methods have been established and protocols are very reliable (for example, DNA/RNA isolation, RT-PCR, in-situ hybridization, anti-body staining and cell injection). All those advantages were taken in account for the consideration of sequencing the entire genome of *Capitella* sp. I by the Joint Genome Insitute. *Capitella* sp. I has a small genome of only 330 Mb, and only 10 chromosomes have been described (Grassle et al. 1987). With the sequenced genome and the following gene annotation, faster results and new techniques are available. Thus, it is also possible to establish *Capitella* sp. I as a new annelid model organism and a new lophotrochozoan model system.

1.2.2 *Platynereis dumerilii*

Platynereis dumerilii has been bred in the laboratory since 1953 without interruption (Fischer and Dorresteijn 2004). It is a marine polychaete annelid with separate genders. Belonging to the family of Nereididae, this polychaete was previously described as cosmopolitan, but this has to do with a mix-up of different species. It was examined and resolved in a study on different isozymes and karyograms of the species *P. dumerilii, P. megalops* and *P. massiliensis* (Jörg 1993). *P. dumerilii* can be found european wide up from the mediterranean sea, over the atlantic coast line all the way north to the south coast of Norway. The worm lives in self-spun living tubes in which it dwells for its entire lifetime of up to two years, occasionally leaving it for the search of food.

Before sexual maturation, genders are indistinguishable. The polychaete grows to a size of up to 40 mm. The body of *P. dumerilii* can be divided into three regions. The prostomium or head possesses one pair of antennae, one pair of palps and four pairs of peristomial cirri. The body region posterior of the head consists of up to 75 morphologically similar appearing segments displaying a homonomous segmentation. The various segments bear a pair of parapodia each equipped with a notopodial and neuropodial set of chaetae. A pair of inner stiff bristles (aciculae) which are moved by the surrounding musculature allow

crawling and swimming movements. The posterior end of *P. dumerilii* or pygidium has a pair of anal cirri containing gland cells and diffusely distributed sensory cells. The subterminal growth zone is located right in front of the pygidium and is responsible for the addition of new segments from the posterior of the animal.

Reproduction takes place by broadcast spawning when animals are sexually mature. For this process, the polychaete has to metamorphose from its atokous immature form to the epitokous reproducing form. Females become yellowish in colour due to the accumulation of oocytes freely floating within the entire body cavity. The males look green to white in the anterior half of the worm because of the high amount of sperms shining through the very thin body wall and the posterior of the body becomes red due to the higher blood flow in this muscle-rich region of the polychaete. When both genders are brought together, females and males communicate by pheromone interaction and spawn by releasing thousands of eggs and innumerous sperm. After fertilization, eggs are surrounded by a jelly coat probably consisting of mucopolysaccharides.

Early cleavage of *P. dumerilii* is spiral and unequal and has been studied intensively in the past up to gastrulation (Dorresteijn 1990; Dorresteijn and Eich 1991; Dorresteijn and Graffy 1993; Dorresteijn et al. 1993) in addition to the early work of Wilson on two close species of *Nereis* (Wilson 1892). After 20h to 24h of development, a planktonic, lecithotrophic trochophore larva hatches from the surrounding egg jelly (Fig. 3A; Fig. 4A1 and 2). A stomodaeum has already formed as well as a prototroch and one pair of larval eyes. The spherical trochophore can be subdivided into an upper part, the episphere, and a lower part, the hyposhere, which are separated by the prototroch. Larvae are positively phototactic. Twelve hours later, the development of the stomodaeum and pharynx is more obvious and the ventral neuroectoderm can be distinguished from the rest of the ectoderm (Fig. 4B1 and 2). First signs of segmentation in form of the first two chaetal sacs are visible (Fig. 4B3 and 4). While the larvae elongate, the paratroch at the posterior end of the hyposhere appears. At 48h of development, two pairs of adult eye anlagen have already formed (Fig. 3B). Elongation of the trochophore proceeds and three pairs of bilateral ciliated bands appear at the posterior of each larval segment (Fig. 4C3 and 4). These three

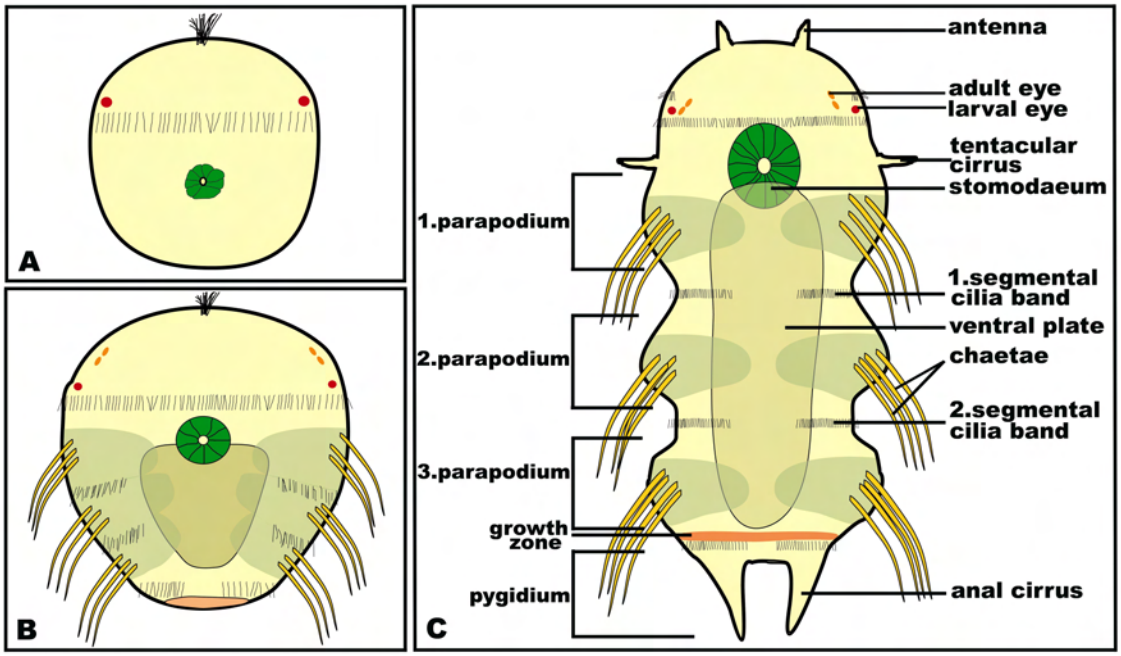


Fig. 3. Schematic of three characteristic developmental stages of *P. dumerilii*. A. 24h – trochophora; apical tuft, mouth, prototroch and a pair of larval. **B.** 48h; metatrochophora with three segment bearing chaetae. The ventral neuroectoderm can be identified as ventral plate and two pairs of adult eyes have been formed. **C.** 72h - nectochaeta; Three pair of parapodia are characteristic for the so called nectochaeta. Larval appendages or anlagen for adult appendages have formed like anal cirri, tentacular cirri or anlagen for the antennae. A-C ventral views.

larval segments have been formed with the internal condensation of the chaetal sacs and the appearance of three pairs of chaetae on each side of the larvae (Fig. 4C1 and 2). The posterior growth zone is established (Fig. 3B) for the formation of the future tritomeres, but is mitotically quiescent for at least two weeks, in contrast to the first three segments, which are called deutomeres due to their formation as primary segments. At 60h of development, protrusion and growth of the chaetae is visible (Fig. 4D1 and 2). The metatrochophore stage is characterized by a positive allometric growth of the hyposheric part of the larvae and the resulting elongation. Besides the stomodaeum, the formation of the proctodaeum anlage is visible as well as the developing gut.

After three days of development, the nectochaeta stage has been reached and is characterized by the presences of three larval segments, the formed parapodia and a further elongated trunk (Fig. 3C; Fig. 4E1 and 2). The anlagen for the antennae, cirri and palps are visible. Over the course of the next few days, the nectochaeta transforms into a juvenile worm by losing its cilia and adopting a benthic life. Small particles of algae can already be eaten by the young worm. After settling, it begins to form a living tube by secreting substances with the glands located in the parapodia. The prostomium, the peristomial segment – a rudimentary larval segment - and the following first segment initially bearing

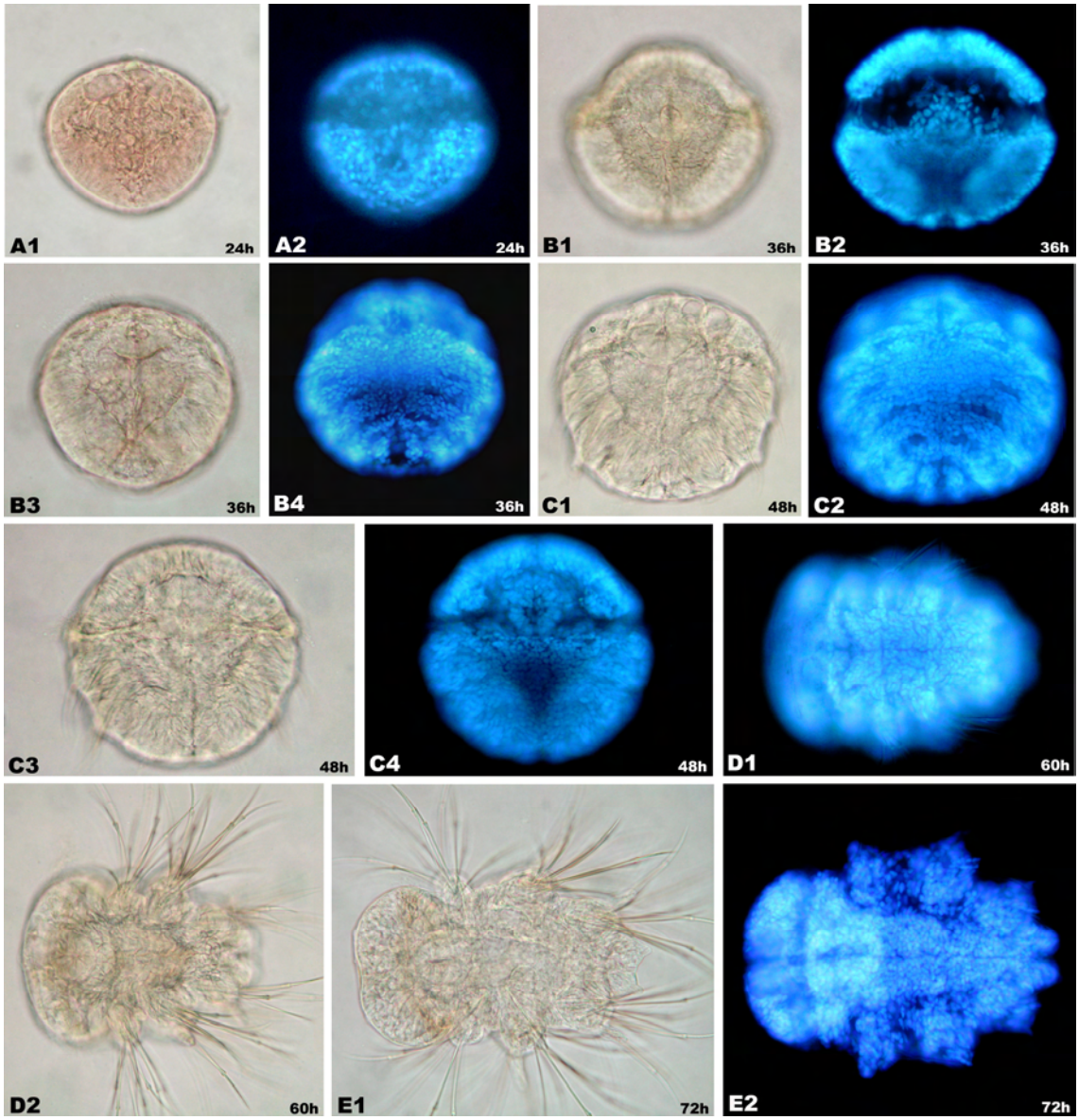


Fig. 4. Larval development of *Platynereis dumerilii*. A1, 2. 24h; mouth and prototroch are visible. **B1-4.** 36h; the first two chaetal sacs are formed. **C1-4.** 48h; the third chaetal sac is formed and the chaetae protrude the larva. **D1, 2.** 60h; the larva elongates and chaetae grow. **E1, 2.** 72h; parapodia are formed. 24-48h: anterior is up, ventral down and dorsal up. 60h-3W: anterior is to the left, ventral down and dorsal up. A1, 2, B1, 2, C3, 4, D1, 2, E1 and 2 are ventral views. B3, 4, C1 and 2 are ventroposterior view. A1, 2, B1-4, C1-4, D1, 2 and E1, 2 are the same animals. A2, B2, 4, C2, 4, D1 and E2 were exposed to Hoechst 33342.

parapodia fuse together to form the head. The integration of larval segments into the head is called cephalization.

Segments are now being added from the so called subterminal growth zone (Fig. 3C). This region is located anterior to the pygidium. Segment proliferation in *P. dumerilii* continues throughout its life time and is asynchronous. The worm grows over the course of the next few weeks and reaches reproductive maturity after three to six months of development. Like in most annelids, the ability to regenerate is immense in *P. dumerilli* and has been

subject of many studies. Regeneration is limited to the posterior addition of segments after amputation. Thus, if anterior segments are cut off the worm dies. Posterior regeneration growth is faster than normal growth. Several segments are simultaneously added during regeneration instead of one segment every five days (Fischer and Dorresteijn 2004). It has been assumed that the developmental program of regeneration is only a faster version of normal addition of segments during juvenile growth, which has still to be verified (de Rosa et al. 2005; Hofmann 1966; Prud'homme et al. 2003). The need for a special cerebral hormone in order to regenerate was shown by prostomium ablation and implantation (Hauenschild 1960).

Altogether, several advantages make *P. dumerilii* a good organims for developmental studies. Reproduction can be controlled in the lab with the help of an 'artifial moon'. Breeding and spawning result in thousands of simultanously fertilized eggs each day and also simultanously developing embryos up to a certain stage of development. *P. dumerilii* is especially well suited for studies of early development because of the transparency of eggs and egg shells. After hatching, positive phototaxis of the larvae makes it easy to collect them. Rapid development of the larvae can be a positive aspect during the course of experiments like cell lineage tracing. Many different methods have been established in the last couple of years like basic molecular techniques (DNA/RNA isolation, PCR, in-situ hybridization), cell lineage tracing and parental RNAi (Ackermann et al. 2005; Arendt et al. 2001; Denes et al. 2007; Rebscher et al. 2007). In the past, many scientists have worked with *P. dumerilii* and related species like *Nereis* creating a huge amount of highly detailed data, especially in the embryonic morphology and development (Dorresteijn 1990; Dorresteijn et al. 1993; Hauenschild 1969; Wilson 1892). Thus, molecular scientists can now refer to it and use this data for identifing structures, mutations, gene expression patterns and results of functional studies and cell lineage tracing.

1.3. Comparison of *Capitella* sp. I and *P. dumerilii*

Table 1 shows the the differences and similarities of larval development, life history and morphology of *Capitella* sp. I and *Platynereis dumerilii* in comparison to each other. Due to their differences in life history, they show divergence in their larval development and morphology. Belonging to the group of polychaetes, both animals also have common characteristics like the presence of a trochophora larva, ciliary bands, the spiral cleavage, and posterior addition of segments from the juvenile stage on until adulthood. Both show so called ancestral features as well as derived characteristics, some of which are still discussed like the ancestry of the parapodia in polychaetes (Purschke 2002; Rouse and Fauchald 1995; Rouse and Fauchald 1997) or the structure of the trochophora larvae (Nielsen 2004; Rouse 1999). It seems to be confirmed that polychaetes show more ancestral features than the second group of annelids, the Clitellates. This latter group includes the Hirudinea and the Oligochaeta which both possess the derived character of a clitellum. On the base of rare and scattered fossils, predictions were made about ancestral features of polychaetes (Conway Morris 1979). Until now, no agreed upon idea of a basal body plan in annelids exists (McHugh 2000). The ancestral features of polychaete larvae are also unknown.

	***Capitella* sp. I**	***Platynereis dumerilii***
Body and life history		
Body plan	Prostomium Segmented thoracic and abdominal body Pygidium	Prostomium Segmented Body Pygidium
Genders	Female/male, hermaphroditic	Female/male
CNS	Two cerebral ganglia as anlagen	One anlage for the brain
Chaetae	Thoracic: Capillary chaetae Abdominal: hooded hooks Neuro-and notopodial rows	Several packs of chaetae in one parapodium incl. one acicula per neuro- and notopodium Chaetae in every segment Morphologically identical
Life history	Benthic, in mud	Benthic, in self-spun tubes
Parapodia	Reduced	Well developed parapodia
Regeneration	Yes, posterior	Yes, posterior
Reproduction	Ø 12 weeks after metamorphosis	Ø 6 months after metamorphosis
Larval development		
Fertilization	External	External, spawning
Egg number	Ø 250/brood tube	Ø 2000-3000/spawn
Size	200 µm	160 µm
Duration	8 days	3 days
Early cleavage	Spiral, unequal	Spiral, unequal
Larval type	Lecithotrophic In brood tube Trochophore	Lecithotrophic Planktonic Trochophore
Ciliary bands	Prototroch Neurotroch Telotroch	Prototroch Paratroch (discontinous)
Phototaxis of larva	Yes (St. 6)	Yes (24h)
Larval segmentation	13 segments	3 segments
Larval segment characteristics	Bilateral belly plates as presumptive segmented tissue for the first 10 segments	Ventral plate as presumptive segmented tissue: 3 segments form almost simultaneously
Larval post. growth	Yes; up to 4 segments	No
Metamorphosis	In one day	Several days between planktonic and benthic stage, gradual
Segment addition in juveniles	1 segment every 3 days	1 segment every 5 days
Eyes	1 pair of larval eyes 1 pair of adult eyes	1 pair of larval eyes 2 pair of adult eyes

Tab. 1. Comparison of life histories, body plans and larval development of *Capitella* sp. I and *Platynereis dumerilii*

1.4 The *Notch* signaling pathway

Strikingly, the Metazoa only rely on a handful of signaling pathways which are indispensable various aspects of their development, including cell differentiation, proliferation, migration or apoptosis. Hedgehog (Hh), Janus kinase/signal transducers and activators of transcription (Jak/STAT), Wnt/Wingless (Wnt), receptor tyrosine kinase (RTK), transforming growth factor-β/Decapentaplegic (TGF-β/Dpp) and *Notch* are key signaling pathways highly conserved within this group of organisms (Cummings 2006; Hurlbut et al. 2007; Pires-daSilva and Sommer 2003). We focused on the *Notch* signaling pathway in this study to concentrate on its involvement in the developmental processes of *Capitella* sp. I and *P. dumerillii* especially during segmentation. *Notch* seems to be involved in a huge variety of networks, diseases, organogeneses and developmental processes and it is connected to other signaling pathways.

This short introduction focuses on the presentation of the canonical conserved *Notch* signaling pathway and its components excluding the exceptions such as repressor CSL-(like *Suppressor of hairless*) or ligand-independent signaling or *hes* transcription without *Notch* (Berechid et al. 1999; Kageyama et al. 2007; Martinez Arias et al. 2002; Mok et al. 2005; Shawber et al. 1996). Signaling is induced by the binding of the DSL-ligand (Delta, Serrate, lag-2) to the *Notch* receptor. Afterwards, *Notch* is cleaved twice proteolytically by a ADAM (Disintegrin and Metalloproteinase) and γ-secretase. The resulting NICD (Notch Intracellular Domain) is transported to the nucleus, where it binds to a CSL (CBF1 (mammalian), Su(H) (*Drosophila*) and Lag-1 (*C. elegans*)) to activate the transcription of target genes such as *hes* genes (Fig. 5).

First, the translational product of the *Notch* gene has to be transformed into a mature heterodimer generated by the proteolytical cleavage of a furin-like convertase. This first modification of the Notch receptor is required for the Notch signaling pathway and has been experimentally demonstrated (Rand et al. 2000). The cleavage occurs in the secretory pathway before binding of the ligand (Blaumueller et al. 1997; Logeat et al. 1998), and afterwards the receptor is presented at the cell surface. The heterodimer is held together by non-covalent interactions preventing the receptor activation in the absence of the ligand. The NECD (Notch extracellular domain) possesses EGF repeats necessary for binding the ligand Delta or Serrate (Rebay et al. 1991). With the binding of the ligand Delta, a signaling site of the Notch receptor in the extracellular domain is exposed

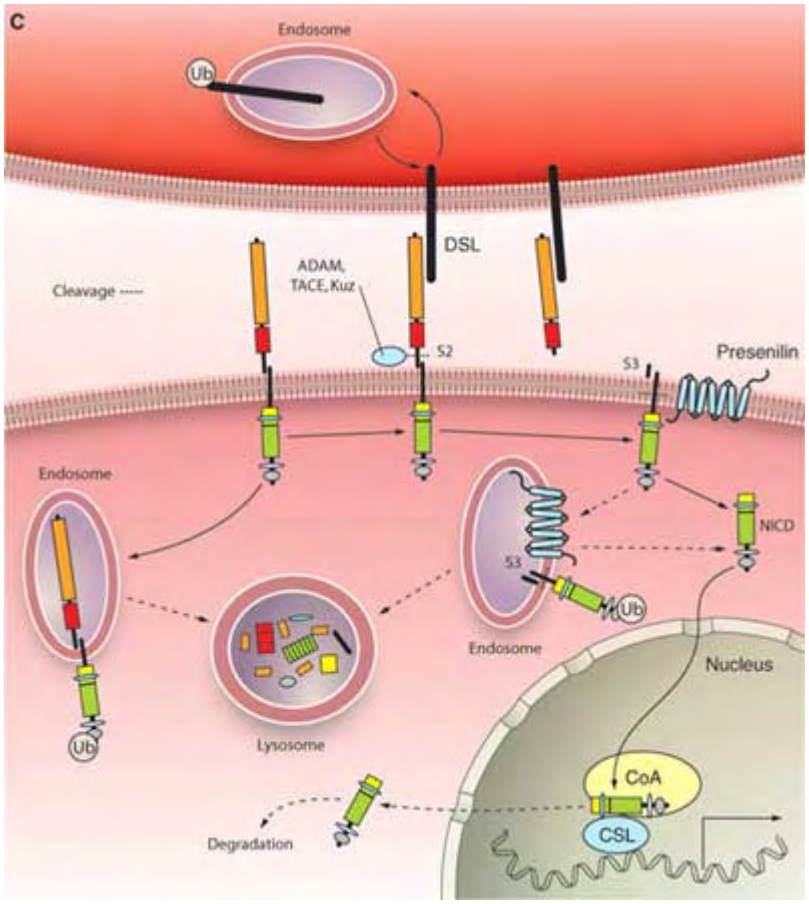


Fig. 5. The *Notch* signaling pathway. The signa-ling cell presenting the Notch receptor on its surface and the donor cell with the Delta receptor display neighboring cells. After binding to Delta, the heterodimer Notch cleaved by a furin-like convertase is processed by a second proteolysis by TACE. The following third cleavage by presenilin produces the NICD which is transported to the nucleus to activate target genes like *hes* by binding to the repressor CSL (modified after Ehebauer et al. 2006).

(Gordon et al. 2007). The presentation of the region is necessary for the following second cleavage of the Notch receptor by the metalloprotease TACE (TNF-α converting enzyme) (Brou et al. 2000), an enzyme belonging to the group of ADAM enzymes resulting in the so called NEXT fragment (Notch Extracellular Truncated). Before the cleavage by the ADAM enzyme can occur, an endocytosis of the Delta-NECD complex into the donor cell (Delta-presenting cell) takes place by physical dissociation of the Notch heterodimer and not by promoting enzymatic dissociation as previously proposed (Nichols et al. 2007; Parks et al. 2000). This process triggers the proteolytic cleavage of the Notch fragment by TACE. Experiments knocking out components required for endocytosis like Neuralized, Mindbomb or shibire/dynamin resulted in an incapabability to activate Notch signaling (Lai et al. 2001; Lai et al. 2005; Lai and Rubin 2001; Seugnet et al. 1997). After the second cleavage, the third proteolysis can occur by the γ-secretase proteolytic complex (Berechid et al. 1999; Ray et al. 1999; Struhl and Adachi 2000; Struhl and Greenwald 1999; Wong et al. 1997) consisting of the four core components presenilin, nicastrin, APH1 (anterior pharynx defective1) and PEN2 (Presenilin enhancer 2) (Wolfe 2006). The catalytic activity of the complex is provided by the enzyme presenilin in vertebrates or

called Sel-12 in *C. elegans* (Gupta-Rossi et al. 2001; Oberg et al. 2001). Mutations in the *Presenilin* gene are known to have the same effect like Notch knock outs and thus, they are indispensable for Notch signaling like all components of the Notch signaling pathway (De Strooper et al. 1999; Ray et al. 1999). Presenilin plays a role in the Alzheimer`s disease where it processes another transmembrane protein (Li et al. 2003; Micchelli et al. 2003; Parks and Curtis 2007).

After the third cleavage the resulting NICD is translocated to the nucleus to interact with CSL (Fortini and Artavanis-Tsakonas 1994; Furukawa et al. 1995; Schweisguth 1995; Tamura et al. 1995). In the absence of the NICD, CSL is a transcriptional repressor by forming multiprotein transcriptional repressor complexes including the corepressors SMRT (silencing mediator of retinoid and thyroid receptors)/N-CoR (nuclear receptor corepressor), Hairless, SPEN or SHARP (SMRT/HDAC-1-associated protein) and CIR (CBF1-interacting corepressor) (Barolo et al. 2002; Hsieh et al. 1999; Kao et al. 1998; Lai 2002; Oswald et al. 2002). The histone deacetylase complexes (HDACs) are also recruited, so that the local chromatin is converted into a transcriptionally silent form. By binding of the NICD to CSL, the repressors are replaced except for the transcriptional coregulator SKIP (Ski-interacting protein), which is usually present during repression and activation of genes (Zhou et al. 2000). When the NICD is bound to CSL, the transcriptional co-activator Mastermind binds to form a ternary complex with both (Fryer et al. 2002; Petcherski and Kimble 2000). Transcription itself is activated by recruitment of the general transcription factors PCAF/GCN5 and CBP/p300 to the complex (Kurooka and Honjo 2000; Wallberg et al. 2002). As a result target genes like *hes, cyclin D1, interleukin-6, CD23* and *myc* are transcribed (Iso et al. 2003; Kovall 2007).

There are several modulators of the Notch signaling pathway such as Numb, Deltex, Disheveled, and Hairless (Panin and Irvine 1998). Another regulator displays the O-fucose specific β-1,3 N-acetylglucosaminyltransferase *fringe*. It was first discovered to play a role in wing development of *Drosophila* (Irvine and Wieschaus 1994). With the glycosylation of Notch, its activation by Delta is potentiated and Notch activation by a second ligand Serrate is inhibited (Fleming et al. 1997; Panin et al. 1997). After the discovery of *fringe*, it was found to be involved in other developmental processes in invertebrates as well as in vertebrates including boundary formation during leg segmentation and somitogenesis (de Celis et al. 1998; Evrard et al. 1998; Irvine and Vogt 1997; Pourquie 2002). Recent results reveal that glycosylation of Notch probably inhibits the formation of the heteromeric

complex between the receptor and Delta forcing Notch to represent a homomeric instead of a heteromeric molecule at the cell surface. This is supposed to enhance the receptivity of Notch to its ligand Delta (Katsube and Sakamoto 2005). How the process of inhibition of Serrate/Jagged and the activation of Delta and its facilitation by *fringe* in the Notch pathway works in detail is a topic of ongoing investigation and controversial discussion. Despite the missing detailed information of *fringe* action during the Notch signaling, it is clear that Notch activation is regulated by *fringe* temporally and spatially (Bruckner et al. 2000; Moloney et al. 2000).

1.5 Evolutionary aspects of the *Notch* signaling pathway and segmentation

As stated earlier, the *Notch* signaling pathway is indispensable for development in animals. In vertebrates, *Notch* and its components are also involved in somitogenesis (see 1.1), a process controlled by the so called segmentation clock. This mechanism is characterized by the transcriptional and translational oscillation of certain genes sweeping through the presomitic mesoderm (PSM) establishing new somites. The process is combined with signaling gradients setting the new location of intersomitic boundaries, called the determination front (Aulehla and Herrmann 2004). With the established pace of oscillating genes, somites are formed in a predictable manner in time and space, specific for each species. On the base of the segmentation clock and determination front, somites are budded off from the PSM in an anterior to posterior direction (Fig. 6). This so called clock and wavefront model was a initially just a theory, but proved to be true with the discovery of the first oscillating expression pattern of *hairy* in the PSM in chicken (Cooke and Zeeman 1976; Palmeirim et al. 1997). The wave of *hairy* expression begins in the posterior of the PSM and sweeps through it until it reaches the anterior end, where it stops and establishes

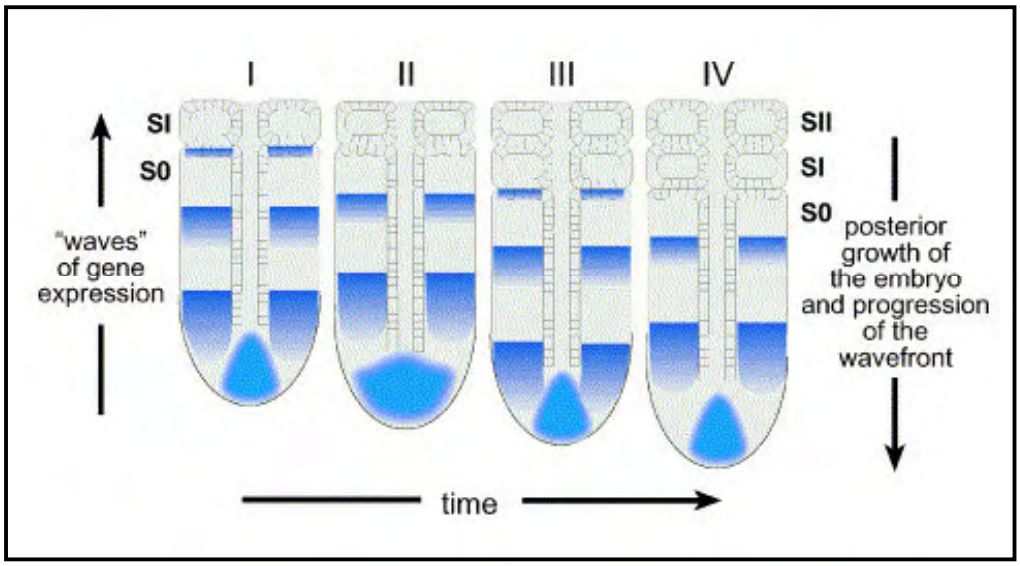


Fig. 6. Clock and wavefront during somitogenesis of vertebrates. The wave of *hairy* expression migrates through the PSM in an posterior to anterior direction, stabilizes at the future posterior border of the somite and a new segment is formed. The determination front established by genes like *fgf8* progresses further towards the posterior of the PSM during somitogenesis to ensure the maintenance of the immature state of the cells in this region. By the interaction of these two factors and also other components and signaling networks, somites are formed in an anterior to posterior progression (modified after Holley and Takeda 2002).

the boundaries of the newly formed somite. Afterwards, several other genes were discovered with a similar cyclic expression pattern mostly belonging to the *Notch* pathway. FGF signaling seems to display the determination front in vertebrates (Sawada et al. 2001). It is expressed at high levels in the posterior and in a lower concentration at the anterior of the PSM where somites can form. FGF is supposed to establish a positional, but also temporal signal for somitogenesis by the polarity of its transcript (Vasiliauskas and Stern 2001).

Notch, *Delta,* and *fringe* are expressed in the PSM. Depending on the organism, some of these genes show cyclic expression patterns including *Delta* and *fringe*, and this cycling appears to be very important for the formation of somites (Hrabe de Angelis et al. 1997; Jiang et al. 1998; Prince et al. 2001). Since *hes* genes are downstream targets of the *Notch* pathway, *Notch* and its components are indispensible for the transcription of *hes/her* genes in vertebrates and also for the cyclic expression of these genes (Bessho et al. 2001; Chen et al. 2005; Jouve et al. 2000; Li et al. 2003; Oates et al. 2005; Serth et al. 2003). Recent research on somitogenesis also revealed that other molecular pathways are involved in the formation of somites, such as the *wnt*-signaling pathway (Aulehla et al. 2003). Recent results discovered a highly coordinated regulation of an organized network of signaling pathways including Notch, FGF/MAPK and Wnt (Dequeant et al. 2006; Mallo 2007).

Mutations of *Notch* genes and components of this signaling pathway such as *fringe* (Evrard et al. 1998; Prince et al. 2001), *Delta* (Dornseifer et al. 1997; Hrabe de Angelis et al. 1997), *Su(H)/rbp-jk* (Oka et al. 1995; Sieger et al. 2003), *presenilin* (Wong et al. 1997) and *hes* genes (Bessho et al. 2001; Takke and Campos-Ortega 1999), result in somitic phenotypes such as uncoordinated formation of somites and fuzzy border formation. The oscillation of cycling genes in *Notch* signaling mutants was also disturbed.

Strikingly, recent studies on the *Notch* signaling and its components *Delta, hairy, Su(H)* and *presenilin* in the basal arthropod *Cupiennius salei* revealed an involvement of *Notch* signaling in the segmentation of this spider (Schoppmeier and Damen 2005; Stollewerk et al. 2003). The *Cs-Delta* and *Cs-Notch* homologues are expressed in the posterior growth zone in stripes and in the newly formed segments in an *engrailed*-like manner being strongly expressed in the posterior part of the segments (see1.1). *Delta* even shows dynamic expression in the posterior growth zone. If this feature of *Delta* expression is due to an oscillation of transcription comparable to the vertebrate *Delta* genes remains to be determined. Functional experiments by RNAi knockdown experiments of *Notch* and *Delta*

result in a malformation of segments including size, shape and width of every segment. Segment borders are also not formed properly. The *hairy* gene, usually expressed in a dynamically pattern of stripes in the growth zone and newly formed segments (Damen et al. 2000), shows a scattered expression after RNAi of *Delta* and *Notch*. This proves that the *Notch* signaling is required for the organization of the striped expression pattern of *hairy* in the spider, and also seems to be a downstream target comparable to the situation in vertebrate somitogenesis (Jouve et al. 2000). Knock-down of *Su(H)* in the spider results in severe segmentation defects. First, after the formation of the third segment, the embryo stops adding segments from the enlarged posterior growth zone. Segments are misshaped and reduced in size and width. Thus, *Su(H)* mutants show more severe defects in the formation of segments than *Notch* mutants. Secondly, *Delta* as well as *hairy* expression are disturbed after RNAi of the spider *Su(H).* Both results are comparable to the vertebrate *Su(H)* mutants (Sieger et al. 2003). *Presenilin* knock out in the spider results in similar defects as those for *Su(H)* and *Delta*, and *hairy* expression is disturbed (Schoppmeier and Damen 2005). These studies show many similarities in the canonical pathway of *Notch* signaling across arthropods and vertebrates and also in the segmentation process of both phyla.

These results are very surprising, because *Notch* and its components do not play any role in the formation of segments in *Drosophila*. This can probably be explained by the derived segmentation mode of *Drosophila* forming segments in a syncytial environment almost simultanously (see 1.1). Cell-cell contacts are not apparent at this stage and thus pathways like *Notch* signaling cannot be utilized for that particular purpose. Most arthropods add segments by the posterior growth zone (see1.1). Due to the functional results in *Cupiennius salei*, a basal arthopod, we can assume a segmentation process in this group, which utilizes the *Notch* signaling pathway as one core component during the formation of segments comparable to vertebrates.

What about the third segmented phylum, the annelids? Do they also utilize the *Notch* pathway and its components like *Delta, hairy, Su(H)* or *fringe* to form segments? The perspective from arthropods were the motivation to examine segmentation of annelids as was already been done for genes like *engrailed, wingless, eve, caudal* and *hedgehog* (Bely and Wray 2001; de Rosa et al. 2005; Prud'homme et al. 2003; Seaver and Kaneshige 2006; Seaver et al. 2001; Song et al. 2004; Weisblat 1983). To gain more insights into the segmentation process of annelids, the expression of *Notch, Delta, hes* homologues, *fringe*

and *Su(H)* during larval development of two different polychaetes, *Capitella* sp. I and *Platynereis dumerilii* was studied in this doctoral thesis. *Notch* and a *hes* homologue have been studied in one annelid so far, the leech *Helobdella* (Rivera et al. 2005; Song et al. 2004). Both genes are expressed in the teloblasts and the segmental founder cells in a dynamic manner. Therefore, these results point towards a common mechanism of segmentation. On the other hand, the leech represents one of the most highly derived annelid clades. Thus, it is inevitable to study the signaling pathway in a more basal group of annelids represented by the polychaetes.

In this study we also want to investigate the diversity of possible functions of the *Notch* signaling pathway in polychaetes. It will be interesting to analyze conserved patterns and possible functions similar in all three phyla such as in neurogenesis or myogenesis. New utilization of this conserved pathway on the basis of the different life histories, development or morphology of the two polychaetes can also be expected. The conservation of the *Notch* pathway, in general, is also one of the central questions. Is it possible to find evidence for a ligand-receptor interaction of isolated *Delta* and *Notch* genes in both polychaetes? Expression data for components involved in the pathway as well as downstream targets like *hes* genes are also very important for an analysis of the signaling and its conservation across polychaetes and the other phyla.

2. Material and methods

2.1 Living material

2.1.1 *Capitella* sp. I colony

A colony of *Capitella* sp. I was maintained in the laboratory of the Kewalo Marine Lab/Honolulu/Hawai'i/USA in incubators at 18°C according to the culture methods developed by (Grassle and Grassle 1976) and described in (Seaver et al. 2005). Culturing occurred in glass bowls with filtered natural sea water FSW. Animals were fed with sieved mud. The extraction of brood tubes was achieved by the sieving of the entire bowl content through a fine-mesh net. Brood tubes were dissected with very fine forceps. The stages of embryos and larvae were determined by eye, then fixed or raised to the desired stage in 35mm Petri-dishes.

2.1.2 *Platynereis dumerilii* colony

A *Platynereis dumerilii* colony was kept at 18°C in an air-conditioned culturing chamber at the Justus-Liebig-University of Giessen, Germany after the culturing methods of Hauenschild (Hauenschild 1969). The origin of the Giessen-colony can be traced back to the original colony of *Platynereis dumerilii* at the University of Mainz, Germany.

2.1.3 Bacterial strains

The following bacterial strains of competent cells were used to amplify plasmids in vivo: DH5α-cells, One Shot TOPO cells with One Shot® TOP10 Chemically Competent E. coli; K4500-01; invitrogen), NEB5α-cells (NEB5α Competent E. coli (High Efficiency) C2987H- New England Biolabs) or XL1-Blue supercompetent cells (Stratagene).

2.2 Chemicals and solutions

2.2.1 Buffers

10% SDS	dissolve 100 g SDS in 1000 ml DEPC-H_2O
10x gel loading buffer	50% Glycerol, 100 mM EDTA (pH 7,5), 1.5 mM Bromophenolblue, 1.9 mM Xylenecyanol in ddH_2O
10x PBS	*Capitella* sp. I: 18.6 mM NaH_2PO_4, 84.1 mM Na_2HPO_4, 1.75 M NaCl in DEPC-H_2O; adjust pH to 7.4 *P. dumerilii*: 1.38 M NaCl, 131.22 mM $Na_2HPO_4 \cdot 7H_2O$, 16.6 mM KH_2PO_4 in DEPC-H_2O
20% Tween –20	200 µl Tween-20 in 1 ml DEPC-H_2O
20x SSC	0.3 M Na citrate, 3 M NaCl in ddH_2O; adjust to pH 7.0 and autoclave
3.7% formaldehyde-solution	1 ml formaldehyde (37 %) in 9 ml FSW
4% paraformaldehyde in NSW	4 ml 16% paraformaldehyde in 12 ml NSW
16% paraformaldehyde-solution	dissolve 8 g paraformaldehyde in 50 ml ddH_2O at 55°C with 3 drops of 10 M NaOH; adjust to pH 7.5
50% glycerol	50 ml glycerol in 50 ml ddH_2O
50x TAE	dissolve 242 g Tris in ddH_2O; add 100 ml 0.5 M Na_2EDTA (pH 8.0) and 57.1 ml glacial acetic acid; adjust to 1 l with ddH_2O
80% Glycerol/ Hoechst	80 ml glycerol in 20 ml PTw, add 50 µl of Hoechst stock solution
Ampicillin stock solution	dissolve 100 mg ampicillin in 100ml ddH_2O
Anti-Dig-AP-antibody solution	1 µl Anti-Dig-AP antibody in 5 ml blocking solution (1:5000)

AP-buffer	100 mM NaCl, 50 mM $MgCl_2$, 100mM Tris (pH 9.5), 0.5% Tween-20 in ddH_2O
AP stop buffer	20 mM Tris (pH 8.0), 5 mM EDTA, 0.1% Tween-20 in ddH_2O
Artificial seawater (ASW)	dissolve 300g „tropic marine“ sea salt in 10 l water, add 10 ml of each stock solution (I-V)
BCIP	50 mg/ml in 100% DMF
Blocking solution	1 ml of 10x Boehringer-Mannheim blocking buffer in 9 ml 1x maleic acid buffer
BSA, 5%	500 mg BSA in 10 ml DEPC-H_2O
CTAB buffer (RNA extraction)	2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2mM NaCl, 0.05% Spermidinetrihydrochloride, 2% β-Mercapto-ethanol
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl (pH 9.5) in ddH_2O
Glycine solution	100 µl glycine stock solution in 10 ml DEPC-H_2O
Glycine stock solution	dissolve 200 mg glycine in 1 ml DEPC-H_2O, aliquote and store at -20°C
Heparin stock solution	50 mg heparin in 1 ml DEPC-H_2O
Hoechst stock solution	1 mg Hoechst 33342 in 1 ml ddH_2O
Hybridization buffer	*Capitella* sp. I: 50% formamide, 5x SSC, 50 µg/ml heparin, 0.1% tween-20, 1.0% SDS, 100 µg/ml salmon sperm DNA in DEPC-H_2O *P. dumerilii*: 50% formamide, 5x SSC, 50 µg/ml heparin, 250 mg Torula-RNA (Sigma) solid, 1.0% Tween-20 in DEPC-H_2O
IPTG	100 mM IPTG in ddH_2O
Lysozyme-stock solution	20 mg/ml in 10 mM Tris-HCl, pH 8.0
Lithiumchloride	10 M in DEPC-H_2O

$MgCl_2$ in seawater	0.37 M $MgCl_2$ in seawater
Maleic Acid Buffer	100 mM maleic acid, 150 mM NaCl in ddH_2O
Methanol-wash-solutions	*Capitella* sp. I: 60% MeOH/ 40% PTw, 30% MeOH/ 70% PTw *P. dumerilii*: 75% MeOH/ 25% PTw, 50% MeOH/ 50% PTw, 25% MeOH/ 75% PTw
NBT	75 mg/ml in 70% DMF/ 30% ddH_2O
PBT	PT, 0.1% BSA, filter sterilize
PCR-buffers	10x ThermoPol reaction buffer: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 10x standard taq reaction buffer: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 10x MasterTaq kit reaction buffer: 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM Mg^{2+}
Plasmidprep-solution I	50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA in ddH_2O, filter sterilize, add lysozym (end concentration 4µg/ml) right before use
Plasmidprep-solution II	0.2 M NaOH, 1% SDS in ddH_2O freshly prepared
Plasmidprep-solution III	8 M NH_4-Ac in ddH_2O, autoclave and store at RT
Posthybridization-solutions	*Capitella* sp. I: 75% hybe/ 25% 2x SSC, 50% hybe/ 50% 2x SSC, 25% hybe/ 75% 2x SSC, 2x and 0.05x SSC, 75% 0.05x SSC/ 25% PTw, 50% 0.05x SSC/ 50% PTw, 25% 0.05x SSC/ 75% PTw *P. dumerillii*: 50% formamide/ 2x SSCT, 2x and 0.2x SSCT
Proteinase-K solution	0.01 mg/ml Proteinase K in PTw

Proteinase-K stock solution	20 mg/ml in DEPC-H_2O
PT	1x PBS, 0.2% Triton X-100 in DEPC-H_2O, filter sterilize
PTw	1x PBS, 0.1% Tween-20 in DEPC-H_2O, filter sterilize
Salmon testes gDNA solution	10 mg/ml in DEPC-H_2O, heat at 95-100°C for 5-10 min and ice-shock for 2 min before adding to hybe
Stock solution I (for ASW)	20 g/l Na_2-EDTA, 200 mg/l $FeCl_3$ in ddH_2O
Stock solution II (for ASW)	2 g/l H_3Bo_4, 200 mg/l Na_2MoO_4, 2.3 g/l $ZnSO_4$, 650 mg/l $MnSO_4$, 6 mg/l $CoSO_4$, 1 mg/l $CuSO_4$ in ddH_2O
Stock solution III (for ASW)	22 g/l KBr, 20 mg/l KI, 6 mg/l LiCl, 60 mg/l RbCl, 3.8 g/l SrCl2, 30 mg/l $AlCl_3$ in ddH_2O
Stock solution IV (for ASW)	100 g/l $NaNO_3$, 20 g/l Na_2HPO_4 in ddH_2O
Stock solution V (for ASW)	20 g/l NaSilicate in ddH_2O
TE-Buffer	10 mM Tris, 1 mM EDTA pH 8.0 in DEPC-H_2O
TfbI-solution	100 mM RuCl, 40 mM $MnCl_2{\cdot}4H_2O$, 30 mM NaAc, 10 mM $CaCl_2$, 15% glycerol in ddH_2O, adjust to pH 5.8 with glacial acetic acid, filter sterilize
TfbII-solution	75 mM $CaCl_2$, 10 mM MOPS, 10 mM RuCl, 15% Glycerol in ddH_2O, adjust to pH 6.8 with KOH, filter sterilize
Triethanolamine-solution	1% TEA in PTw
Washing buffer	0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween-20 in ddH_2O
X-Gal	40 mg/ml in 100% DMSO

2.2.2 Oligonucleotides

2.2.2.1 Degenerate primers

2.2.2.1.1 *Capitella* sp. I

Delta: Delta2F2in 5‘-GAY GAY VHV TTY GGN CAY TWY WSN TG-3‘

Delta2R2in/out 5‘-CAR YAN ARN CCN CCC CAN CCY TC-3‘

Delta2R1in 5‘-ANG TNC CRT GNA NRC ANC CNG G-3‘

Fringe: Fringe-fw1 5‘-GAY RTN TTY ATM DSN GTN AAR ACN AC-3‘

Fringe-fw2 5‘-TTY TGY CAY KTN GAY GAY GAY AAY TAY G-3‘

Fringe-rv1 5‘-KNS WNA DRC ARA ANC CNG C-3‘

Fringe-rv2 5‘-CCN CCN TGN GCR AAC C-3‘

Hairy/ hes: HES-leech-fw 5‘-MGI GMI MGI ATN AAY RAN TSN YT-3‘

h-bw1 5‘-CTG NAR RTT CTG NAR RTG YTT NAC-3‘

h-bw2 5‘-GTY WTY TCN ARD ATR TCN GCY TTY TC-3‘

Notch: anknotchFout 5‘-GNM GNA CNC CNY TNC AYG C-3‘

CDC3.2 5‘-CAR TGN ARN GCN SMY TTN CC-3‘

anknotchF1in 5‘-TNG CNR TNG ARG GNA TGB TNG ARG-3‘

2.2.2.1.2 *Platynereis dumerilii*

Hairy/hes: Csh-fw1 5’-AAR CCN ATH ATG GAR AAR MGN MG-3’

for h-bw1 and h-bw2 see *Capitella* sp. I

Mef2: Psn-fw1 5`-TAY GGN GCN MAR CAY GTN AT-3’

Psn-bw0 5’-C YTG NGC NGT YTC NAC-3’

Psn-bw1 5’-GG NAR RTA YTT DAT RAA NAC-3’

Su(H): Su(H)-fw1 5’-CAY GCN AAR GTN GCN CAR-3’

Su(H)-Dr-fw2 5’-GTN AAR ATG TTY TAY GGN AA-3’

Su(H)-Dr-bw3 5’-DAT RTA RAA NGC NCC CCA YTG-3’

Su(H)bw2 5’-TG NSW NAC NGG RTC RTC NGC-3’

2.2.2.2 RACE-primers and specific primers

2.2.2.2.1 *Capitella* sp. I

Delta: ccDelta5out 5‘-GAA TGG CAC GAT GCA GTG-3‘

ccDelta3out 5‘-GCA GTT GCA TTC CCA CTC-3‘

ccDelta5inII 5‘-GCA AGG CAA CCT GTG CGA CCA ATG CAT C-3‘

ccDelta3in 5‘-GAT GCA TTG GTC GCA CAG GTT GCC TTG C-3‘

ccDelta3‘ext. 5‘-ACG AAT GTT CAT CTC AAC CGT GC-3‘

ccDelta3‘ext.II 5‘-TCG TCA CCG TCG CAG ACC-3‘

Hairy: cchairy5out 5‘-CCA TCA ACT CAA AGT TCT CG-3‘

cchairy3out 5‘-CTA ACT TGG AAA ATC TCG CG-3‘

cchairy5in 5‘-CCA TCA ACT CAA AGT TCT CGT TTT GGA TGC TCT C-3‘

cchairy3in 5‘-CTA ACT TGG AAA ATC TCG CGC TAT CTT TTT TGA GAG CAT C-3‘

Fringe: ccfringe5out 5‘-GAA TAC GCG AGC TCT CG-3‘

ccfringe3out 5‘-CTT GTC GCC CAT CTG-3‘

ccfringe5in 5‘-GAG CTC TCG TGC GCT TGC TGA GGA AAT ACA AG -3‘

ccfringe3in 5‘-GTC GCC CAT CTG CAG CGG ATC ACT GAG GCT CC-3‘

Notch: 5‘-Notch 5‘-GAT CTG CTC AAC GCC AAA GCG GAG GTC AAT GCC ACC G-3‘

3‘Notch-GSPI 5‘-CAT GTC TCA CCC TGC CTA CCC TGT GTC GC-3‘

3‘Notch-GSPII 5‘-CCG CTT ACC TCT AGC GCT C-3‘

2.2.2.2.2 *Platynereis dumerilii*

Actin: ActinRT-fw 5’-AGA TCT GGC ATC ACA CCT TCT AC-3’

ActinRT-rv 5’-CTC GTG GAT ACC AGC GGA TTC-3’

Delta: 5’Delta-out 5’-CTG CGA AGG ATC TTA CAC CTG CGA ATG CCT TGC GCG-3’

3’Delta-out 5’-CCG GTG TAG CCT GGT CGG CAG TGG CAC CG-3’

5`Delta-in 5'-CGG ATT ACC AAT GTC AGT GTC CTC CAG GGT TCC GTG GC-3'

3'Delta-in 5'-GGT TCT CCT CGC ACG AAG TGG CGC TGC TCT CGC-3'

3'Delta-ext. 5'-CGC TAA CTG TCA TTT GCA CGA CG-3'

Hairy: 5'hairy-out 5'GCC CAT AAT GGA GAA GCG TCG ACG AGC CAG AAT-3`

3'hairy-out 5'-CGA AAA TGT CTG CCT TCT CAA GTT TGG AAT ATC TGG AGG-3'

5'hairy-in 5'-CCC TCA ACA TGC TGA AGA CTC TTG TAT TGG ATG CTT TG-3'

3`hairy-in 5'-CAA AGC ATC CAA TAC AAG AGT CTT CAG CAT GTT GAG GG-3'

3'hairy,ext. 5'-CAA GCC CTA ACA GTA GCA GAA G-3'

Hes: 5'hes-out 5`-GCG AAG AGC TCG AAT CAA TGC CAG CCT CAC GGA AC-3'

3'hes-out 5'-CAT CTT GTG GCG CCT CGT GCC CTC T-3'

5'hes-in 5'-GCT CGA ATC AAT GCC AGC CTC ACG GAA CTC AAG ACA C-3'

3'hes-in 3'-CAT CCA GGA GAA GTG TCT TGA GTT CCG TGA GGC TGG C-3'

3'hes,ext. 5'-CAG AAC AAT CTC AAC AGC AGT G-3'

Mef2: 3'mef2-out 5'- CAC ACA CTA ACT GGC CAC CCA CTC GGA AAG GC-3'

3'mef2-in 5'-CGC TTC ATA CTC GGC CCG TCA AAG TCC GTT GG-3'

5`mef2ext. 5`-GAT CAT CTT CAA CTC TGC CAA C-3'

Notch: Notch2-fw 5'-GAG ACG ACA TAT TTG CCG TGT TC-3'

Notch2-rv 5'-CAG TTG GCT CCA CTC TTA CAG-3'

Su(H): 5'Su(H)-out 5'-CGG TTA CGG TCT CAA ACA GTC AGC ACC CGG TAT CTA C-3'

3'Su(H)-out 5'-CGT AGA ACT AGC GTG GAA GTT TCC TCC CTC CAC GTG-3'

5`Su(H)-in 5'-GTC AGC ACCCGG TAT CTA CAC GTG GAG GGA GGA AAC TTC-3'

3'Su(H)-in 5'-GTA GAT ACC GGG TGC TGA CTG TTT GAG ACC GTA ACC G-3'

3'Su(H)ext 5'-GAA GGAATTG GGT CCC GTC-3'

2.2.2.3 Standard primers

GeneRacer Oligo dT 5'-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG T(24)-3'

M13E 5'-GTA AAA CGA CGG CCA GTG-3'

M13F(-20) 5'-TGT AAA ACG ACG GCC AGT-3'

M13R(-20) 5'-CAG GAA ACA GCT ATG ACC -3'

Nested Universal Primer A: 5'–AAG CAG TGG TAT CAA CGC AGA GT–3'

SP6 5'-CAT TTA GGT GAC ACT ATA G-3'

T3 5'-AAT TAA CCC TCA CTA AAG GG-3'

T3RAC 5'-CTG GAG CTC CAC CGC GGT GGC-3'

T3RAC-LOW 5'-CTG GAG CTC CAC CGG GGT G-3'

T7 5'-TAA TAC GAC TCA CTA TAG GG-3'

T7RAC 5'-GAC TCA CTA TAG GGC GAA TTG GGT ACC CGG-3'

T7RAC2 5'-GAC GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA G-3'

T7RAC2-LOW 5'-GAC GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA-3'

Universal Primer Mix A:Long 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3'

Short 5'-CTA ATA CGA CTC ACT ATA GGG C-3'

2.2.3 Kits and enzymes

- 100bp DNA ladder — Genecraft, Promega
- 1kb DNA ladder — Genecraft, Promega
- Advantage 2 Polymerase Mix — Clontech, Takara

• Anti-Digoxygenin alkaline phosphatase-coupled antibody	Roche
• DIG RNA Labeling Mix, 10x conc.	Roche
• DIG-11-UTP	Roche
• DNAse (RNAse free)	NEB
• DNAzol	Molecular Research Center
• DNeasy tissue kit	Qiagen
• dNTP-mix	NEB, Peqlab, Promega
• EcoRI	Fermentas
• FastPlasmid Mini Kit	Eppendorf
• First-strand cDNA synthesis kit	Amersham Pharmacia Biotech
• GeneRacer RACE Ready cDNA Kit	Invitrogen
• Lysozym	Sigma
• MasterTaq Kit	Eppendorf
• MEGAscript SP6 Kit	Ambion
• MEGAscript T7 Kit - 40 rxns	Ambion
• MinElute Gel Extraction Kit	Qiagen
• Omniscript RT Kit	Qiagen
• PeqGOLD RNAPure	Peqlab
• pGEM-T Easy Vector System I	Promega
• Proteinase K	GibcoBRL, Sigma
• RNAse A	Sigma
• RNeasy Mini Kit	Qiagen
• SMART RACE cDNA Amplification Kit	Clontech, Takara
• SP6-Polymerase	Roche
• T7-Polymerase	Roche
• *Taq* DNA Polymerase	NEB, Promega

- TRIReagent — Molecular Research Center
- TOPO TA Cloning kit — Invitrogen
- Wizard PurePlasmid kit — Promega

2.2.4 Bacterial culture media

LB-medium	10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 10 g NaCl in 900ml ddH_2O, adjust to pH 7.0 with 10 M NaOH, adjust volume to 1 l with ddH_2O, autoclave and store at RT
LBamp-plates with IPTG and X-Gal	add 15 g Bacto-Agar to 1 l LB-medium, autoclave, cool down to 50°C, add 1 ml of ampicillin stock solution, mix by swirling and pour into sterile petri-dishes, store plates at 4°C after solidification before use, pipette 40 µl IPTG-stock and 40 µl X-Gal stock onto plate
SOB-medium	20 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 0.5 g NaCl, 2.5 ml 1 M KCl in 900 ml ddH_2O, adjust to pH 7.0 with 10 M NaOH and adjust volume to 990 ml with ddH_2O, autoclave, add 10 ml sterile 1 M $MgCl_2$ just before use
SOC-medium	add 20 ml sterile 1 M glucose to 1 l SOB-medium just before use
TB phosphate	2.31 g KH_2PO_4, 12.54 g K_2HPO_4 in 90 ml ddH_2O, autoclave and store at RT
TB-medium	12 g Bacto-Tryptone, 24 g Bacto-Yeast extract, 4 ml glycerol in 900 ml ddH_2O, autoclave and cool to 60°C, add 100 ml of sterile 10x TB phosphate, store at RT

2.2.5 Cloning vectors

The pGEM-Teasy cloning vector (Promega) and the TOPO-TA vector (Invitrogen) were used for the plasmid cloning (vector maps in supplemental material).

2.3 Technical equipment

2.3.1 Electrophoresis

- E-BOX Video-Documentationssystem — Peqlab
- Electrophoresis Power Supply — EC Apparatus, Consort
- Foto/Prep Transilluminator — Fisher, ETX
- Gel electrophoresis chamber — Ellard Instruments Ltd., Peqlab
- Kodak EDAS 290 IC Mac 110VAC — Kodak

2.3.2 PCR-Cycler

- Cyclone-Gradient — Peqlab
- Cyclone 25 — Peqlab
- Mastercycler gradient — Eppendorf

2.3.4 Centrifuges

- Eppendorf 5415D Microcentrifuge — Eppendorf
- Eppendorf 5415R Microcentrifuge — Eppendorf
- Mikro-20 — Hettich Zentrigufen
- Mikro-22R — Hettich Zentrigufen
- Personal Microcentrifuge — US Scientific Inc.

2.3.5 Incubators and shakers

- 15-460-2S Economy Water Bath — Fisher

• Big Shot, 115VAC Hybridization Oven	Boeckel Scientific
• Biometra Compact Line OV4	Biometra
• Hybridization Oven	
• Certomat H	Braun Biotech international
• Incubator Memmert	Memmert
• Incubator Tv15	Memmert
• Julabo 19; EC-BRU/PU	Julabo
• Julabo 4-HC Water Bath	Julabo
• LabLine Model 2000 Open Air Shaker	LabLine
• LabLine Model 4000 Incubator Shaker	LabLine
• Precision Economy Incubator	Thermo Scientific
• Rotamax 120, shaker	Heidolph Instruments
• Rotilabo-Block Heater H-250	Roth
• VCM-LSI Block Heater	VCM
• Vortex Labuco L46	Labuco
• VWR Signature low-temperature B.O.D. Incubator	VWR

2.3.6 Optical equipment

• Axioskop 2 mot	Zeiss
• Axioskop 2 plus	Zeiss
• Stereomicroscope MZ 16	Leica
• Cold-light source KL1500LCD	Schott
• Nikon CoolPix 4500	Nikon
• Nikon CoolPix 990	Nikon
• Olympus-BX41TF	Olympus
• Olympus-BX51TF	Olympus
• Stemi 2000	Zeiss

2.3.7 Others

- Basic Bio pH MV Temp Meter — Denver Instrument Comp.
- BioMate 3 Spectrophotometer — Thermo Spectronic
- Constructa energy freezer — Constructa
- Fisher Adjustable Flame Burners — Fisher
- Hot plate stirrer model L-81 — Lab
- Magnetic stirrer model L-71 — Laborbrand
- pH 211 microprocessor pH meter — Hanna Instruments
- Sartorius Basicplus Series Top Loading Balance — Satorius
- Sartorius Corporation Competence CP — Satorius
- Design Analytical Balance
- Satorius CP1243 Analytical Balance — Satorius
- Satorius Iso-9001 Top Loading Balance — Satorius
- Siemens starcollection electronic-freezer — Siemens
- Smart-Spec 3000 — Biorad
- Superaka-freezer — Privileg

2.3 Methods

2.3.1 Fixation of embryonic and larval stages

2.3.1.1 *Capitella* sp. I

After sieving, brood tubes were transferred into 35 mm petri dishes and dissected. Embryos were pipetted into a new plastic dish for further treatment. For stages before and around stage 4, a 1:1 mixture of 0.1 M sucrose: 0.25 M sodium citrate was added for 3 min. Treatment was terminated by washing with FSW. Moving stages (stage 5-9 and juveniles) were immobilized with 0.37 M $MgCl_2$ in FSW for 5-15 min depending on the movement of the larvae. After pretreatment, all stages were fixed in 3.7% formaldehyde in FSW for 30 min to 1 h at RT or o/n at 4°C. Fixation was stopped by washing with 1x PBS three times for 5 min each. Embryos were transferred into 100% MeOH and washed 3 times with 100% MeOH. Animals were stored in 100% MeOH at -20°C until further treatment.

2.3.1.2 *P. dumerilii*

Larvae older than 48 hours were relaxed in 0.37 M $MgCl_2$ for 10 min. Animals were transferred into 1.5 ml eppendorf tubes and $MgCl_2$/ NSW mixture or NSW was exchanged with 4% PFA in NSW or 3.7% formaldehyde in NSW. Embryos were fixed for 1-4 hours at RT with shaking on a rocker or o/n at 4°C without shaking. Fixative was removed and substituted by 1x PTw. Three wash steps with 1x PTw for 5 min each followed. After transferring into 100% MeOH and washing, embryos were stored in 100% MeOH at -20°C until further treatment.

2.3.2 Genomic DNA isolation

Before isolation of genomic DNA, worms were starved for up to 1 week in an extra dish containing either FSW or ASW.

2.3.2.1 DNeasy tissue kit

Adult worms were transferred into a 1.5 ml eppendorf tube. 180 µl ATL buffer was added to the animal. The tissue was ground with a plastic mortar until it had dissolved completely. Digestion of the tissue was induced by adding 20-50 µl Proteinase K stock to the ATL buffer. An incubation at 56°C for 1-4 hours or o/n followed. An optional digestion with 4µl RNase (100 mg/ml stock) for 5 min at RT can be done. To precipitate the DNA, 200 µl AT buffer and 200µl 100% EtOH were added and mixed by vortexing. The contents of the tubes were transferred onto a DNeasy mini spin column with a collection tube. The first centrifugation step was performed at 8000 rpm for 1 min and the flow through plus collection tube discarded. The column was washed by adding 500 µl AW1 buffer, centrifugated and the flow-through was discarded. A second wash step was performed with 500 µl AW2 buffer and centrifugation at 14,000 rpm for 3 min to dry membrane. The mini spin column was transferred into a new microcentrifuge tube. To elute the DNA, 100 µl AE buffer was pipetted onto column and centrifuged at 8000 rpm for 1 min. The last step was repeated with the same 100 µl AE buffer to increase the DNA yield. The final concentration of the isolated DNA was determined by spectrophotometry.

2.3.2.1 DNAzol

1-2 worms were transferred into an 1.5 ml eppendorf tube. The 10x volume of DNAzol was added to the 1x volume of the animal (~500-700 µl DNAzol). The tissue was homogenized with a plastic pestle. 100 µg/ml Proteinase K were added, mixed thoroughly by brief vortexing and placed at RT for ~3 hours or o/n. After incubation for several hours, the tube was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and transferred into a new 1.5 ml eppendorf tube. To precipitate the DNA, 0.5 ml EtOH were added for each ml of DNAzol used. The tube was inverted several times and incubated for 10-15 min. Afterwards the visible DNA strands were transferred into a new 1.5 ml tube. To collect all DNA, the EtOH-DNAzol mixture in the old tube was centrifuged again for 5 min at 5000 rpm at 4°C and the supernatant was removed. The resulting pellets were washed 3 times with 1 ml of 100% EtOH. The EtOH was removed completely and the pellet dried for 5-10 min at RT or 37°C. The DNA was resuspended in 100 µl TE buffer (pH 8.3), specced out and stored at -20°C.

2.3.3 RNA isolation

For all three methods an optional DNase treatment with subsequent recovery of the RNA with the RNeasy kit can be performed depending on the following applications. This DNase digestion and resuspension of the pellet were done after the isolation of the RNA. 3 µl DNase (1U/ µl) and 3 µl 10x DNase buffer were added to the RNA sample. After a 10 min incubation at 37°C, a heat inactivation of the enzyme followed at 75°C for 10 min. EDTA was added to a final concentration of 5 mM to protect the RNA from degradation during the enzyme inactivation. The sample was purified with the RNeasy Mini Protocol for RNA Cleanup.

The concentration of each sample was determined by spectrophotometry. The quality and integrity of the RNA structure was examined by running a small amount on a 1% agarose gel.

2.3.3.1 RNeasy

Desired larval stages were collected and pipetted into a 1.5 ml tube. 600 µl RLT buffer containing 6 µl freshly added β-Mercaptoethaol were used for 30 mg tissue. The embryos were homogenized in RLT buffer with a pestle and incubated at RT for 10 min. A syringe with a needle was used to shear the DNA by passing lysate through the needle. The sample-buffer mixture was centrifuged at 14,000 rpm for 3 min. The supernatants were transferred into a new 1.5 ml eppendorf tube. One volume of 70 % RNase free EtOH was added to the lysate and mixed by inverting. The sample was applied to a new RNeasy column and centrifuged at 10000 rpm for 15 sec. The flow through was discarded, 700 µl of RW1 buffer added and the centrifugation step was repeated. The same centrifugation steps were performed with RPE buffer twice. The RNA was eluted by pipetting 30-50 µl RNase free water onto column and centrifugation at 10,000 rpm for 1 min.

2.3.3.2 CTAB protocol (Zeng and Yang 2002)

A RNA isolation protocol with CTAB was modified after Zeng and Yang to recover RNA from *P.dumerilii* stages younger than 24 hours and unfertilized eggs, which possess a mucose- and polysaccharide rich coat. The CTAB buffer was prewarmed to 65°C. Desired stages were collected or dissected in case of the unfertilized eggs and transferred into a container. Liquid nitrogen was added. The embryos or eggs were ground using a mortar.

The powder was transferred into tubes, 750 µl prewarmed CTAB buffer added and incubated at 65°C for 10 min with vigorous shaking several times. A centrifugation step followed at 12000 rpm for 10 min. The viscous supernatant was transferred to a new tube. By adding an equal volume of chloroform-isoamylalcohol, the sample was reextracted. The lysate was centrifuged again under the same conditions. This reextraction step was performed three times to lose more and more of the polysaccharides. Afterwards, the aqueous phase was spun at maximum speed (14000 rpm) for 20-30 min at RT. The insoluble material was discarded. The RNA was precipitated by adding 0.25 volume of 10 M LiCl to the supernatant, mixed well and stored at 4°C o/n. The sample was recovered by centrifugation at maximum speed for 30 min at 4°C. The resulting pellet was washed three times with 100 µl 70% RNase free EtOH. The RNA was dried and resuspended in 30 µl DEPC-H_2O or TE buffer.

2.3.3.3 TRI Reagent

To 50-100 mg of the sample 1 ml of TRI Reagent was added. The tissue was homogenized with a pestle and incubated at RT for 5 min. The homogenate was supplemented with 100 µl BCP or 200 µl chloroform. The tube was shaked vigorously for 15 sec and stored at RT for 10-15 min. A centrifugation step followed at 14000 rpm for 15 min at 4°C to separate the mixture into a lower phenol-chloroform phase with DNA, interphase and upper aqueous phase containing the RNA. The aqueous phase was transferred into a new 1.5 ml eppendorf tube. The RNA was precipitated by addition of 500 µl isopropanol. After incubation at RT for 10 min, a centrifugation step at 14000 rpm for 8 min at 4°C followed. The resulting RNA pellet was washed with 1 ml 75 % EtOH and a subsequent centrifugation at 9000 rpm for 5 min at 4°C. After removing the EtOH, the pellet was dried at RT for 3-5 min. The pellet was resuspended in 50 µl RNase free water.

2.3.4 Degenerate primer PCR

PCRs with the purpose of "fishing" out a new gene of the organisms genome or transcriptome were performed with different templates.
Genomic DNA (*Capitella* sp. I and *P. dumerilii*) was used at a concentration around 100 ng/µl for each 25 µl PCR-reaction.

The cDNA-library of *Capitella* sp. I was used for degenerate primer PCR as well as for RACEs (see 2.3.5). This template is an amplified lambda bacteriophage library. The plasmids are prepped after mass excision. All fragments are in pBluescript vector (see supplemental material). 100 ng/µl were used as template concentration in each PCR.
cDNA generated with reverse transcriptase was often used as a template for PCR. Different kits were used for cDNA synthesis.

2.3.4.1 First-strand cDNA synthesis kit (Amersham Pharmacia Biotech)

COMPONENTS	1X
10x buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNaseOUT Recombinant RNase Inhibitor	1 µl

The mixture was incubated at 65°C for 5 min to denature the RNA. It was placed on ice immediately for 5 min. The following mixture was prepared and 9 µl were added to the RNA/primer mix.

COMPONENTS	SAMPLE
up to 5 µg total RNA	up to 8 µl
10 mM dNTP mix	1 µl
Oligo dT (0.5 µg/ml)	1 µl
DEPC-treated water	up to 10 µl

The mix was incubated at 42°C for 2 min, 1µl of SuperscriptIIRT was added and incubated for 50 min at 42°C. The reaction was terminated at 70°C for 15 min. 1µl of RNaseH was added and incubated for 20 min at 37°C. The mixture could now be stored at -20°C. Depending on the quality of RNA, reaction time and enzyme, the amount of cDNA per PCR reaction varied.

2.3.4.2 Omniscript RT Kit (Qiagen)

The following 20 µl were incubated at 37°C for 60 min and stored at - 20°C for further applications.

COMPONENTS	1X
10x buffer RT	2 µl
20 mM dNTP mix	2 µl
Oligo-dT primer (10 µM)	2 µl
RNase Inhibitor (10 U/µl)	1 µl
Omniscript RT	1 µl
RNase-free water	x µl
Mixed RNA (up to 2 µg)	x µl

2.3.4.3 SMART-RACE cDNA (see 2.3.5.2)

For some PCRs 1-2 µl of the SMART-RACE cDNA were used as a template.

2.3.4.4. Degenerate primer PCR

Degenerate primers were designed with the help of sequence alignments of conserved domains, structures or regions in the genes wanted. The alignments were generated with MacVector 7.2.2.2 and already known sequences from different organisms.

Several kits were used to perform PCRs, but the endconcentration of the needed components for each PCR reaction (25 µl) are mostly the same (see following table):

COMPONENTS	ENDCONCENTRATION
10x buffer	1 x
25 mM/10 mM dNTP mix	200-250 µM
taq-polymerase (5 U/µl)	1 U/25 µl
25 mM Mg (Cl_2, Ac or So_4)	1.5-6 mM
Primer	0.4-2 µM
5x taq-master (if used)	1 x
ddH_2O	x µl
Template	x µl

Variations in the template concentration, the template itself, annealing temperatures, primer- and $MgCl_2$ concentrations and different PCR programs made it possible to amplify the resulting genes in this thesis. The following PCR program is the basic program, which was used during the lab work with minor and major variations in the annealing temperature (depending on the primers), number of cycles and elongation times (depending on the expected fragment size).

Initial denaturation	95°C	5 min	
Denaturation	95°C	30 sec	n cycles
Annealing	40-70°C	30 sec	
Elongation	72°C	30 sec-3 min	
Final elongation	72°C	5 min	
Storage	4°C	∞	

Resulting products were analyzed by running an agarose gel with the appropriate agarose concentration (1.3-2.2 % agarose) and stained with 20 µg/ml EtBr. Bands with the expected size were excised and cloned (see 2.3.6 and 2.3.7) into vectors for amplification and analysis.

2.3.5 RACE-PCR

After the recovery of a fragment by degenerate primer PCR, the additional sequence of the transcript was obtained by RACE-PCR.

Specific primers were designed based on the isolated gene fragments. Two approaches were used, which will be described in the following parts.

2.3.5.1 RACE with *Capitella* sp. I cDNA-library

As mentioned above, the library was introduced into pbluescript vectors. Specific primers, partly with high annealing temperatures for optimized PCR conditions, were designed based on the vector sequence. They are located on both sides of the MCS of the vector comprising the inserts. With outer and inner primers, gene and vector specific primers in both directions, 5`and 3` RACE PCRs were performed to obtain the complete ORFs.

2.3.5.2 SMART-RACE

SMART RACE was performed with SMART-RACE cDNA. The mechanism behind the method as well as the primer design are described in detail in the manual of the BD SMART-RACE kit.

The 5`- and 3`-cDNAs were synthesized with the following components:

5`-RACE-cDNA	3`-RACE-cDNA
1-3 µl total RNA (up to 1 µg)	1-3 µl RNA (up to 1 µg)
1 µl 5`CDS-primer	1 µl 3`CDS-primer A
1 µl BD SMART II A Oligo	
→ add sterile H2O to a final volume of 5 µl	
→ mix and incubate at 70°C for 2 min	
→ cool on ice for 2 min	
add the following to each tube: 2 µl 5X First-Strand Buffer 1 µl DTT (20 mM) 1 µl dNTP Mix (10 mM) 1 µl BD PowerScript Reverse Transcriptase	
10 µl total volume	

Tubes were vortexed briefly to mix, spun down and incubated at 42°C for 1.5 hours. The resulting product was diluted with 100 µl TE-buffer and stored at -20°C for several months.

The RACE-PCRs were performed using the following components for the mastermix:

COMPONENTS	1X
PCR-Grade Water	17.25 µl
10X BD Advantage 2 PCR Buffer	2.5 µl
dNTP Mix (10 mM)	0.5 µl
50X BD Advantage 2 Polymerase Mix	0.5 µl
total volume	**20.75 µl**

These components were mixed thoroughly with 1.25 µl of 5`or 3`SMART-RACE-cDNA, 2.5 µl UPM and 0.5 µl GSP. Single-Primer controls and negative controls were also added to the experiment. The following PCR program was used for amplification:

Denaturation	95°C	30 sec	
Elongation	72°C	3 min	
Denaturation	95°C	30 sec	
Annealing	70°C	30 sec	5 cycles
Elongation	72°C	3 min	
Denaturation	95°C	30 sec	
Annealing	68°C	30 sec	20 cycles
Elongation	72°C	3 min	
storage	4°C	∞	

A secondary PCR was performed using 2.5µl of a 1:50 dilution of the first PCR product as a template, 0.5 µl GSP and 0.5 µl nested universal primer. The following program was used for amplification:

Denaturation	95°C	30 sec	
Annealing	40-70°C	30 sec	15-20 cycles
Elongation	72°C	3 min	
storage	4°C	∞	

Resulting products were analysed by running an agarose gel and stained with 20 µg/ml EtBr. Bands were excised and cloned (see 2.3.6. and 2.3.7.) into vectors for amplification and analysis.

2.3.6. PCR clean up

Bands of expected size and RACE-PCR bands with reasonable size were excised out of the gel. The DNA isolation was performed with the MinElute Gel Extraction Kit (Qiagen)

following the manufacturer protocol. The resulting eluate was used for ligation into a cloning vector.

2.3.7. Cloning

2.3.7.1 Ligation

PCR fragments were ligated into the listed vectors (see 2.2.5) with the following pipetting schemes:

pGEMTeasy

COMPONENTS	1X
vector	1 µl
ligase	1 µl
2x buffer	5 µl
PCR-product	3 µl
total volume	**10 µl**

TOPO

COMPONENTS	1X
Vector	0.5 µl
salt solution	1 µl
PCR-product	up to 4.5 µl
total volume	**6 µl**

The ligation reaction was incubated o/n at 4ºC to optimize the results.

2.3.7.2 Transformation

The plasmid with the ligated PCR-product was transformed into competent cells (see 2.1.3.). The transformation protocol was adapted depending on the cells and the plasmid. The basic protocol was performed as follows: 25-50 µl of competent cells were thawn on ice. Up to 5 µl of the ligation mix were added to the competent cells. The tube was flicked gently and incubated on ice for 30 min. Cells were heat shocked for 30-90 sec and placed on ice for 2 min. 250-500 µl SOC-medium or LB-medium were added. The cells were incubated at 37ºC for 1 hour at 150 rpm. Identification of positive clones was performed with blue/white screening (Sambrook and Russell 2000). On two LB-amp/ IPTG/ X-Gal plates 50 and 200 µl were spread out and placed into the incubator upside down at 37ºC o/n (12 to 16 hours). White colonies were picked onto new LB-amp plates and also incubated o/n at 37ºC.

2.3.7.3 Chemically competent cells

Besides using commercial available cells, competent cells were also made in the lab with the modified Hanahan protocol. Cells were streaked out onto a LB-plate containing no antibioticum or an antibioticum other than ampicillin depending on the resistence of the cells. Plates were incubated o/n at 37ºC and one colony of that plate was used for inoculating into 4 ml sterile SOB medium. 4 x 0.5 ml of this o/n culture were again inoculated into 4 x 50 ml SOB medium and incubated at 37ºC at 250 rpm. Cultures grew until an OD between 0.350 and 0.600 was reached. Then, the bacteria were transferred into prechilled 50 ml conical tubes and chilled on ice for at least 15 min. The tubes were centrifuged at 300 rpm for 12 min at 4ºC. The supernatant was removed and the cells were resuspended in 16 ml TfbI medium by gently pipetting up and down. Cells were incubated on wet ice for 15 min to 1 h. A centrifugation step at 3000 rpm for 12 min at 4ºC followed. The supernatants were removed and the cells resuspended in 2 x 7 ml TbfII. The tubes were incubated on wet ice for 15-30 min. The cells were shock frozen with liquid nitrogen as 50-100 µl aliquots and stored immediately at -80ºC.

2.3.7.4 Insert check

To check the correct size of the insert in the transformed plasmid, a plasmid PCR or a plasmid digestion with restriction enzymes can be performed. In this work, an insert PCR was done with the same PCR conditions as described in 2.3.4. The primer pairs M13F (-20), M13R(-20) and SP6, T7 were used and the elongation of the PCR program was adapted to the expected insert size plus MCS. The PCR products were run out on a gel and the size of the insert was determined.

2.3.8 Plasmid preparation

Single colonies containing a plasmid with the correct insert size were inoculated into 5 ml of LB or TE- medium with 100 µl/ml ampicillin and incubated o/n at 37ºC at 250 rpm. After that, a plasmid preparation was performed with various methods (see 2.3.7.1-2.3.7.3). The final plasmid concentration was determined by spectrophotometry or estimated by running a 1.0 % agarose gel.

2.3.8.1 FastPlasmid Mini Kit (Eppendorf)

The plasmid preparation was performed following the manual with the following modifications. An extra 1 ml of 20 mg/ml Lysozym in Tris-HCl was added to the lysis buffer besides the provided lysozym and RNAse. After dissolving the bacteria cell pellet by vortexing for 30 sec, an incubation step of 5-10 min followed. Before the elution of the plasmid DNA, the tube containing the column and the buffer were incubated for 5 min at RT.

2.3.8.2 Wizard PurePlasmid kit (Promega)

The preparation was performed using the "Plasmid Purification Without a Vacuum Manifold" protocol.

2.3.8.3. "Home made" kit (Kotchoni et al. 2003)

Up to 3 ml o/n culture were transferred into a 2 ml tube and centrifuged for 5 min at 5000 rpm. The supernatants were removed carefully and the pellet was resuspended in 200 µl of plasmid-prep solution I (see 2.2.1) containing 4 µg/ml lysozym. Lysis took place for 5 min at RT. 400 µl of freshly prepared plasmid-prep solution II had to be added to the lysed cells and then mixed well by inverting gently four to six times. 200 µl of plasmid-prep solution III were added and mixed by pipetting up and down. The mixture was incubated on ice for 5 min. The white precipitate containing genomic bacterial DNA, cell debris and proteins had to be removed by centrifugation at 10000 rpm for 5 min. The clear supernatant was transferred into a new tube and 600 µl isopropanol were added and mixed by inversion. The mixture was incubated for 10 to 15 min and centrifuged at 10000 rpm for 5 min to obtain a pellet containing the plasmid DNA. The pellet was washed with 400 µl 70% EtOH. After centrifugation and removal of the supernatants, the pellet was air-dried for 10-30 min and resuspended in 50 µl 10 mM Tris-HCl. An additional RNase digestion (1 µl RNAse (10 mg/ml) for 5 min at 37 ºC) is optional.

2.3.9 Sequencing and sequence analysis

The sequencing of plasmids was done by three companies: Genegateway (California, USA), Macrogen (Seoul, Korea) and Seqlab (Göttingen, Germany).

The obtained sequences were polished by removal of MCS-sequences of the vectors used and the resulting insert data was entered into the BLAST database. A comparison to other listed genes in the Genbank database (NCBI) was made. If the desired gene had been obtained, the sequences were aligned with MacVector 7.2.2 and a consensus sequence was created. The conceptual translation of resulting sequences yielded the predicted ORFs of the desired genes. All sequences used in the alignments and phylogenetic analysis were downloaded from Genbank (see 7.).

2.3.10 Glycerol stocks

Clones with plasmids and the desired inserts were archived as glycerol stocks. The colonies, if older than three months, were streaked out again on a LB-amp plate and incubated o/n at 37ºC. 1 ml of LB medium containing 100 µl/mg ampicillin was inoculated with the colony and incubated at 250 rpm for 8 h at 37ºC. Tubes were labeled thoroughly. 250 µl culture and 125 µl sterile 50% glycerol were mixed gently and stored at -80ºC.

2.3.11 Phylogenetic analysis

The alignment of 43 bHLH-domains, orange domains and WRPW-motive from various *hes* genes (*hairy* and *Enhancer of split*) and *twist* genes (see 7.) for phylogenetic analysis of the *CapI-hes* genes and *Pdu-hes* genes was created in MacVector 7.2.2. with CLUSTALW. A neighbour-joining tree as well as a maximum parsimony tree (Swofford 2002), both with 1000 bootstrap repetitions, were constructed with PAUP4.0b10. The Bayesian tree values were calculated by MrBayesV3.0mac (Huelsenbeck and Ronquist 2001) with the fixed wag-model. A majority consensus tree was generated from 9501 trees, representing 950,000 stable generations.

2.3.12 Whole mount in-situ hybridization (wmish)

2.3.12.1 Probe template

The direction of the inserts in the plasmids was checked and an antisense (either SP6 or T7) was chosen for the probe synthesis.

2.3.12.1.1 PCR product

A plasmid PCR was performed, described in detail in 2.3.4 and 2.3.6.4, to amplify and obtain the pure insert. The total volume amounted 4 x 50 µl instead of 1 x 25µl to obtain a higher concentration of isolated PCR product, which is required for the probe synthesis. After performing the insert PCR, the product was run out on a gel and the following PCR clean up (see 2.3.6) yielded the template for the probe synthesis (see 2.3.12.2).

2.3.12.1.2 Plasmid digestion

To linearize the plasmid for the RNA transcription, a digestion with suitable restriction enzymes was set up. The correct enzyme was chosen with the help of the vector map, which shows the restriction enzyme cutting sites in the plasmid . The following reaction was set up:

COMPONENTS	1X
Plasmid	3 µg
restriction enzyme	1 µl (1U)
10x buffer	2 µl
ddH_2O	x µl
total volume	**20 µl**

The reaction was incubated for 2 h at 37ºC. The restriction enzyme was inactivated by heat shock at 72ºC for 10 min. The linearization was checked on a gel in comparison to unlinearized plasmid. The linearized plasmid was precipitated by adding 2 µl NaAc and 44 µl 96% EtOH to the digestion. The mixture was placed at -80ºC for 1 h or o/n at -20ºC. After centrifugation at 14000 rpm for 30 min at 4ºC, the resulting pellet was washed with 100 µl 70% EtOH. The tube was centrifuged again at 4ºC for 5 min at 14000 rpm and the pellet air-dried. Then the plasmid was resuspended in up to 14 µl TE-buffer and used for in-vitro transcription.

2.3.12.2 Probe synthesis

The RNA-probes for wmish were synthesized with two different kits according to manufacturers instructions. After probe synthesis the resulting RNA was run out on a

agarose gel and the concentration was determined by spectrophotometry. The probe was diluted with hybe-buffer to a final concentration of 50 ng/µl and stored at -20ºC.

2.3.12.2.1 MEGAscript high yield transcription kit (Ambion)

Depending on the orientation of the original sequence in the vector, the T7 or SP6 kit was used. The following components were mixed thoroughly and placed at 37ºC for 4 to 6 hours:

COMPONENTS	1X
10x buffer	1 µl
ATP solution	1 µl
CTP solution	1 µl
GTP solution	1 µl
UTP solution	0.68 µl
Labeled DIG-11-UTP	2.1 µl
template (PCR clean up 2.3.11.1.1)	x µl (0.5 µg)
Enzyme mix (SP6 or T7)	1 µl
RNasefree H_2O	x µl
total volume	**10 µl**

1 µl of TURBO DNAse (2 U/µl) was added and incubated for another 15 min at 37ºC. To precipitate the RNA, 25 µl $LiCl_2$ solution and 40 µl RNase-free water were added to the mix and placed at -20ºC for 30 min or o/n. The tube was centrifuged at 10000 rpm for 15 min at 4ºC. The resulting pellet was washed twice with 150 µl RNase-free EtOH and resuspended in 50 µl TE-buffer.

2.3.12.2.2 DIG RNA Labeling Mix (Roche)

The following components were mixed and placed at 37ºC for 2 h:

COMPONENTS	1X
linearized plasmid or PCR product	x µl (1 µg or 100-200 ng)
10x DIG RNA labeling mix	2 µl
10x transcription buffer	2 µl
ddH_2O, RNAse-free	x µl (depending on template concentration)
RNA polymerase (SP6 or T7) (20 U/µl)	2 µl
total volume	**20 µl**

2 µl of RNAse-free DNAse were added and incubated at 37ºC for 15 min. The reaction had to be stopped by adding 2 µl 0.2 M EDTA (pH 8.0) to the RNA mix. Precipitation of the RNA was obtained by pipetting 2.5 µl 4 M $LiCl_2$ and 75 µl prechilled 100% EtOH to the reaction and placing the tube at -80ºC for 30 min or o/n at -20ºC. RNA was recovered by centrifugation at 4ºC for 15 min at 14000 rpm, washed twice with 50 µl 70% EtOH and resuspended in 50 µl RNase-free water.

2.3.12.3 Blots

2.3.12.3.1 Dot blot

A dot blot with the previously synthesized probes was performed to verify the determined concentration of the probes by spectrophotometry (see 2.3.11.2) with the help of a standardized labeled DIG-RNA control. Various concentrations of the control RNA (10 ng/µl) and the samples (dilution of 1:5 to a final concentration of 10 ng/µl like control RNA) were set up (1:10, 1:100, 1:1000, 1:10000) and 1 µl of each sample and dilution were spotted onto a nylon membrane. The RNA was fixed to the membrane by UV crosslinking (30 sec into UV cross linker) or baking (30 min at 120ºC or 2 h at 80ºC). The membrane was transferred into a plastic container and washed twice with washing buffer for 10 min.

All steps were performed under shaking if not stated otherwise. A 1:5000 anti-DIG-AP (Rôche) antibody dilution in blocking solution was added to the probe and incubated for 30 min at RT. The membrane was washed 3 x 5 min with washing buffer and 3 x 5 min with detection buffer at RT. The colour substrate solution was prepared by using 4.5 µl/ml NBT and 3.5 µl/ml BCIP in detection buffer. The staining solution was then added to the

membrane and developed in the dark for 1-2 min without shaking. The reaction had to be checked every min to avoid overstaining. After developing, the staining reaction was stopped by washing with ddH_2O or AP-stop buffer. The membrane was dried o/n at RT on a piece of whatman paper.

2.3.12.3.2 Southern blot

A southern blot was performed to check the binding of the probe to the right target, even though it is DNA in this blot instead of RNA in the whole mount in situs. I also verified the incorporation of DIG-RNA into the probe with this method. The isolated PCR clean up (see 2.3.11.1.1), which was template for the probe used in this southern blot, was spotted onto the membrane besides a second PCR clean up from a different gene and a control RNA as a positive control to check the antibody binding and NBT/BCIP reaction. The DNA and RNA were fixed to the membrane with the same methods described in 2.3.11.3.1. Then, the membrane was transferred into a plastic bag containing prewarmed hybridization buffer. The plastic bag was sealed and placed into a 65ºC water bath for 30 min. Meanwhile, the RNA-probe for the hybridization was prepared. 50 µg/ml probe were added to a tube with 50µl ddH_2O. The tube was placed at 100ºC for 10 min and put on ice immediately. The denatured probe was transferred into a tube containing 3.5 ml hybridization buffer. The prehybridization buffer was discarded and the probe was added to the membrane. The plastic bag was incubated o/n at 65ºC.

After hybridization, the membrane was washed twice with 2x SSC and 0.1% SDS for 30 min at RT under shaking. The membrane was transferred into a plastic bag again and washed twice with 0.1% SSC and 0.1% SDS for 15 min each wash at 65ºC. After subsequent shaking for 2 min at RT in a petri dish with washing buffer, blocking solution was added to the membrane and blocked for 30 min at RT on a rocker. The membrane was then incubated in a 1:5000 dilution of the anti-DIG-AP antibody in blocking solution for 30 min and washed twice for 15 min with washing buffer. The membrane was equilibrated in detection buffer for 3 min and then it was incubated in staining solution (4.5 µl/ml NBT and 3.5 µl/ml BCIP in detection buffer) for 2-3 min until the spots came up. The membrane was developed until the desired intensity of the spots was obtained and stopped by washing several times with ddH_2O. The membrane was air-dried o/n at RT on a piece of whatman paper.

2.3.12.4 Wmish-*Capitella* sp. I protocol

All washes are with 500 µl each wash 5 min at RT on rocker unless stated otherwise. PTw, PBS and PBT concentrations are always 1x. The protocol was used for both organisms, *Capitella* sp. I and *P. dumerilii*.

Embryos were transferred to 24 well plates and rehydrated with 60% MeOH/ 40% PTw, 30% MeOH/ 70% PTw and 4 PTw washes. Larvae and juveniles were digested with 10 ng/µl Proteinase K for 2-15 min depending on the stage and organism without rocker. The reaction was stopped by two PTw/Glycin (2 mg/ml) washes. Larvae were incubated in 1% triethanolamine in PTw and 3 µl/ml acetic anhydride. After 5 min, 1.5 µl acetic anhydride per well were added and incubated for another 5 min. After 2 washes in PTw, the animals were refixed in 3.7% formaldehyde in PTw for 30 to 60 min at RT. Embryos were washed 5x with PTw and then incubated at 80ºC for 10 min in the hybe oven. After equilibration for 10 min at RT in hybridization buffer, the animals were transferred to new preheated hybe buffer and placed at 65ºC for at least 4 h or better o/n.

Prehybridization can be performed in 24-well plates as well as in 1.5 ml eppendorf tubes placed in a rack.

The used probes were diluted to a final concentration of 1-6 ng/µl (depending on the gene and quality of the probe) in 100-500 µl hybridization buffer. The sample was denatured for 10 min at 85ºC and added to the embryos after removing the prehybridization buffer. The plate or tubes were placed at 65ºC for 1 to 2 days.

The probes were removed and the embryos washed 1x 5 min and 1x 20 min with prewarmed hybridization buffer at 65ºC. The following post-hybridization washes were performed:

10 min 75% hybe/ 25% 2x SSC at 65ºC

10 min 50% hybe/ 50% 2x SSC at 65ºC

10 min 25% hybe/ 75% 2x SSC at 65ºC

10 min 100% 2x SSC at 65ºC

2x 30 min 0.05x SSC at 65ºC

5 min 75% 0.05x SSC/ 25% PTw at RT

5 min 50% 0.05x SSC/ 50% PTw at RT

5 min 25% 0.05x SSC/ 25% PTw at RT

10 min 100% PTw at RT

The embryos were washed 5x with PBT and then incubated in blocking buffer for at least 1 h at RT. Larvae were transferred into Anti-Dig-AP-antibody solution and placed on a rocker o/n at 4ºC.

After the incubation o/n, the animals were washed 7 to 8 times with PBT for 10 min each wash. The embryos were then washed 3 times for 5 min in AP buffer and developed in AP substrate solution (containing 6.6 or 4.5 µl/ml NBT and 3.3 or 3.5 µl/ml BCIP respectively, depending on the organism *Capitella* sp. I or *P. dumerilli*) until desired coloration had been reached. The reaction was stopped by washing 3 times with AP stop buffer. The embryos were equilibrated with 80% Glycerol in PTw o/n and stored at 4ºC.

2.3.12.5 Wmish-*Platynereis dumerilii* protocol

All washes were performed with 500 µl each wash (24-well plate) or 3ml (6-well plate) 5 min at RT on rocker unless stated otherwise. PTw concentration was always 1x.

Embryos were transferred into a 6 or 24-well plate and rehydrated with 75% MeOH/ 25% PTw, 50% MeOH/ 50% PTw, 25% MeOH/ 75% PTw and 2 washes with PTw were done. The Proteinase K digestion (10 ng/µl) was performed for 5-10 min depending on the developmental stage without shaking. The reaction was stopped by rinsing the embryos in PTw/ glycine (2mg/ml) solution twice. Embryos were refixed in 4% PFA in PTw for 20 min and washed five times with PTw. The animals were transferred into 1.5 ml eppendorf tubes and prehybridized in prewarmed hybridization buffer for 1-2 h 65ºC. Meanwhile, 1-6 µl of the desired probes in 100 µl hybridization buffer were denatured for 10 min at 85ºC. The prehybridization buffer was replaced with the probe containing hybe buffer and the tubes were incubated o/n at 65ºC in the water bath or the hybe oven.

The hybridization buffer was replaced by 50% formamide/ 2x SSCT for 30 min at 65ºC. The wash was done twice. Embryos were washed once in 2x SSCT for 15 min at 65ºC and twice for 30 min each wash in 0.2x SSCT at 65ºC. The animals were blocked in 5% sheepserum/ PTw at RT for 1-2 h and incubated in 200 µl preabsorbed anti-DIG-AP antibody (1:2000 dilution) in PTw o/n at 4ºC afterwards. Embryos were transferred into 6-well plate or 24-well plate and washed six times 10 min each wash in PTw. After equilibration with 2 washes 5 min each in SB buffer, embryos were transferred into SB substrate solution containing 4.5 µl/ml NBT and 3.5 µl/ml BCIP. They were developed

until desired staining had been reached, stopped by washing several times with PTw and transferred to 80% Glycerol/PTw for storing at 4ºC in the dark.

Preabsorption of the anti-DIG-AP-Fab fragment antibody

Mature *P. dumerilli* females were fixed like described and stored in 100% MeOH. Animals were rehydrated by washing 3 times 5 min each wash in PTw and homogenized with a pestle. The volume was adjusted to 1ml PTw and 10 µl anti-DIG-AP antibody were added. The tube was incubated o/n at 4ºC on a rocker. The debris was spun down and the supernatant sterile filtered (0.2µm pore size of syringe). The last step was repeated once and the antibody solutions were combined and adjusted to 20 ml with PTw for a final concentration of 1:2000 anti-DIG-AP antibody. The preabsorbed antibody was stored at 4ºC for up to 3 months.

2.3.13 Wmish analysis

All embryos and juveniles were mounted in 80% Glycerol/ PTw containing 1 µg/ml Hoechst for nuclei tracing. The expression of genes was analyzed with a Zeiss Axioscope 2 plus microscope as well as an Olympus-BX41TF and recorded digitally with a Nikon CoolPix 4500 camera.

3. Results

3.1 The ligand *Delta*

3.1.1 *Delta* in *Capitella* sp. I

A 218 bp putative *Delta*-fragment was isolated by seminested PCR using the primers Delta2Fin, Delta2R2in/out and Delta2R1in (see 2.2.2.2.1) and a mixed stage cDNA library of *Capitella* sp. I (see 2.3.4) as template. After performing 5' and 3` RACE PCRs (see 2.3.5), a 2744 bp sequence was isolated containing a complete predicted 2352 bp ORF, which translates into a 784 amino acid long protein. The resulting gene, which we call *CapI-Delta*, possesses several typical characteristics of *Delta* genes (Fig. 7A) (Fleming 1998), all indespensible for the function of *Delta* like the signal peptide, a DSL region, nine EGF repeats, a transmembrane region and an intracellular domain (Fig. 7B).

To analyze the spatial and temporal distribution of the *CapI-Delta* transcript, whole mount in situ hybridizations were performed for *Delta* as well as for all other named genes in *Capitella* sp. I and *Platynereis dumerilii*.

The first signs of *CapI-Delta* expression can be detected in early stage 4 embryos in the developing belly plates (Fig. 8A1 and A2). The transcription of *CapI-Delta* in the presumptive segmented tissue on both sides of the larvae expands rapidly over time (Fig. 8B and C1, bracketed arrows). At stage 5, it is located in the segmented and unsegmented part of the belly plates (Fig. 8C1 and C2, bracketed arrows). The ventral view of stage 5 embryos shows the even distribution of *CapI-Delta*, which at later stages changes into a periodic distribution.

The broad bilateral expression transforms into a two row expression (Fig. 8F, arrows) late stage 5, of which the ventral row is more prominent than the dorsal one. The dot-like expression in the presumptive chaetal sac anlagen becomes stronger at stage 6 (Fig.8. G, arrows). The lateral rows (Fig. 8H1, arrows) of segmentally arranged expression domains (Fig. 8H1, small arrows) are even more refined at late stage 6, right before the chaetae are formed. The two-row expression of discrete spots is also clearly visible in a ventral view (Fig. 8H2, arrows). An upcoming higher concentration of *CapI-Delta* transcript in the

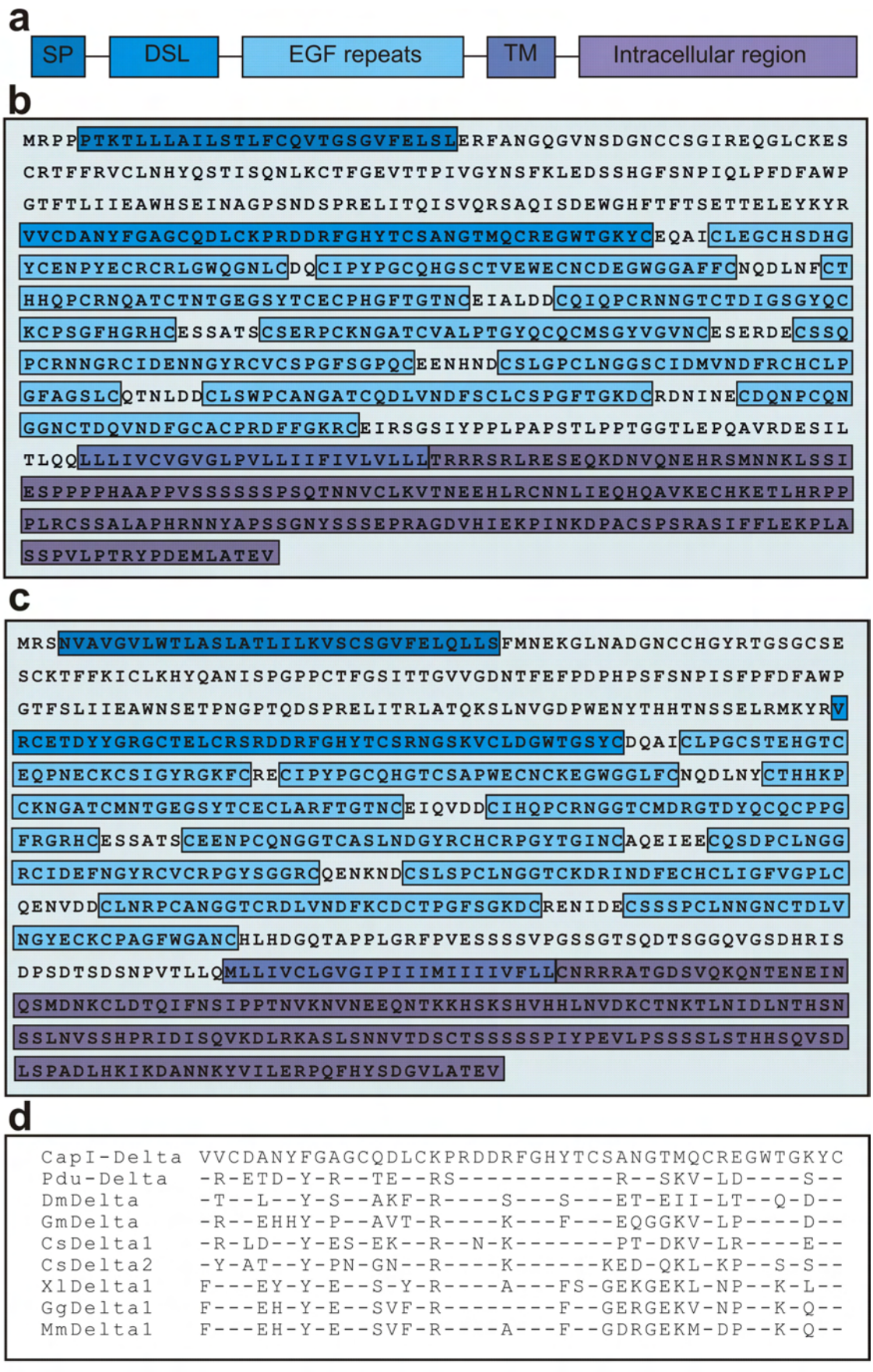

a
SP
DSL
EGF repeats
TM
Intracellular region
b
MRPPPTKTLLLAILSTLFCQVTGSGVFELSLERFANGQGVNSDGNCCSGIREQGLCKES
CRTFFRVCLNHYQSTISQNLKCTFGEVTTPIVGYNSFKLEDSSHGFSNPIQLPFDFAWP
GTFTLIIEAWHSEINAGPSNDSPRELITQISVQRSAQISDEWGHFTFTSETTELEYKYR
VVCDANYFGAGCQDLCKPRDDRFGHYTCSANGTMQCREGWTGKYCEQAICLEGCHSDHG
YCENPYECRCRLGWQGNLCDQCIPYPGCQHGSCTVEWECNCDEGWGGAFFCNQDLNFCT
HHQPCRNQATCTNTGEGSYTCECPHGFTGTNCEIALDDCQIQPCRNNGTCTDIGSGYQC
KCPSGFHGRHCESSATSCSERPCKNGATCVALPTGYQCQCMSGYVGVNCESERDECSSQ
PCRNNGRCIDENNGYRCVCSPGFSGPQCEENHNDCSLGPCLNGGSCIDMVNDFRCHCLP
GFAGSLCQTNLDDCLSWPCANGATCQDLVNDFSCLCSPGFTGKDCRDNINECDQNPCQN
GGNCTDQVNDFGCACPRDFFGKRCEIRSGSIYPPLPAPSTLPPTGGTLEPQAVRDESIL
TLQQLLLIVCVGVGLPVLLIIFIVLVLLLTRRRSRLRESEQKDNVQNEHRSMNNKLSSI
ESPPPPHAAPPVSSSSSSPSQTNNVCLKVTNEEHLRCNNLIEQHQAVKECHKETLHRPP
PLRCSSALAPHRNNYAPSSGNYSSSEPRAGDVHIEKPINKDPACSPSRASIFFLEKPLA
SSPVLPTRYPDEMLATEV
c
MRSNVAVGVLWTLASLATLILKVSCSGVFELQLLSFMNEKGLNADGNCCHGYRTGSGCSE
SCKTFFKICLKHYQANISPGPPCTFGSITTGVVGDNTFEFPDPHPSFSNPISFPFDFAWP
GTFSLIIEAWNSETPNGPTQDSPRELITRLATQKSLNVGDPWENYTHHTNSSELRMKYRV
RCETDYYGRGCTELCRSRDDRFGHYTCSRNGSKVCLDGWTGSYCDQAICLPGCSTEHGTC
EQPNECKCSIGYRGKFCRECIPYPGCQHGTCSAPWECNCKEGWGGLFCNQDLNYCTHHKP
CKNGATCMNTGEGSYTCECLARFTGTNCEIQVDDCIHQPCRNGGTCMDRGTDYQCQCPPG
FRGRHCESSATSCEENPCQNGGTCASLNDGYRCHCRPGYTGINCAQEIEECQSDPCLNGG
RCIDEFNGYRCVCRPGYSGGRCQENKNDCSLSPCLNGGTCKDRINDFECHCLIGFVGPLC
QENVDDCLNRPCANGGTCRDLVNDFKCDCTPGFSGKDCRENIDECSSSPCLNNGNCTDLV
NGYECKCPAGFWGANCHLHDGQTAPPLGRFPVESSSSVPGSSGTSQDTSGGQVGSDHRIS
DPSDTSDSNPVTLLQMLLIVCLGVGIPIIIMIIIIVFLLCNRRRATGDSVQKQNTENEIN
QSMDNKCLDTQIFNSIPPTNVKNVNEEQNTKKHSKSHVHHLNVDKCTNKTLNIDLNTHSN
SSLNVSSHPRIDISQVKDLRKASLSNNVTDSCTSSSSSPIYPEVLPSSSSLSTHHSQVSD
LSPADLHKIKDANNKYVILERPQFHYSDGVLATEV
d
CapI-Delta VVCDANYFGAGCQDLCKPRDDRFGHYTCSANGTMQCREGWTGKYC
Pdu-Delta -R-ETD-Y-R--TE--RS----------R--SKV-LD----S--
DmDelta -T--L--Y-S--AKF-R----S---S---ET-EII-LT--Q-D--
GmDelta -R--EHHY-P--AVT-R----K---F---EQGGKV-LP----D--
CsDelta1 -R-LD--Y-ES-EK--R--N-K-------PT-DKV-LR----E--
CsDelta2 -Y-AT--Y-PN-GN--R----K------KED-QKL-KP--S-S--
XlDelta1 F---EY-Y-E--S-Y-R----A---FS-GEKGEKL-NP--K-L--
GgDelta1 F---EH-Y-E--SVF-R--------F--GERGEKV-NP--K-Q--
MmDelta1 F---EH-Y-E--SVF-R----A---F--GDRGEKM-DP--K-Q--

Fig. 7a. General arrangement of the conserved regions in a *Delta*-protein. SP - signal peptide; DSL - Delta-Serrate-lag2-region; TM – transmembrane region. **7b. *CapI-Delta* amino acid sequence.** Colours of the labeled regions correspond to the colours in 7a. **7c. *Pdu-Delta* amino acid sequence.** Colours of the labeled regions correspond to the colours in 7a. **7d. Alignment of the DSL-regions including *CapI-Delta* and *Pdu-Delta*.** Dashes indicate identical amino acids. Dm-*Drososphila melanogaster*; Gm-*Glomeris marginata*; Cs-*Cupiennius salei*; Xl-*Xenopus laevis*; Gg-*Gallus gallus*; Mm-*Mus musculus*.

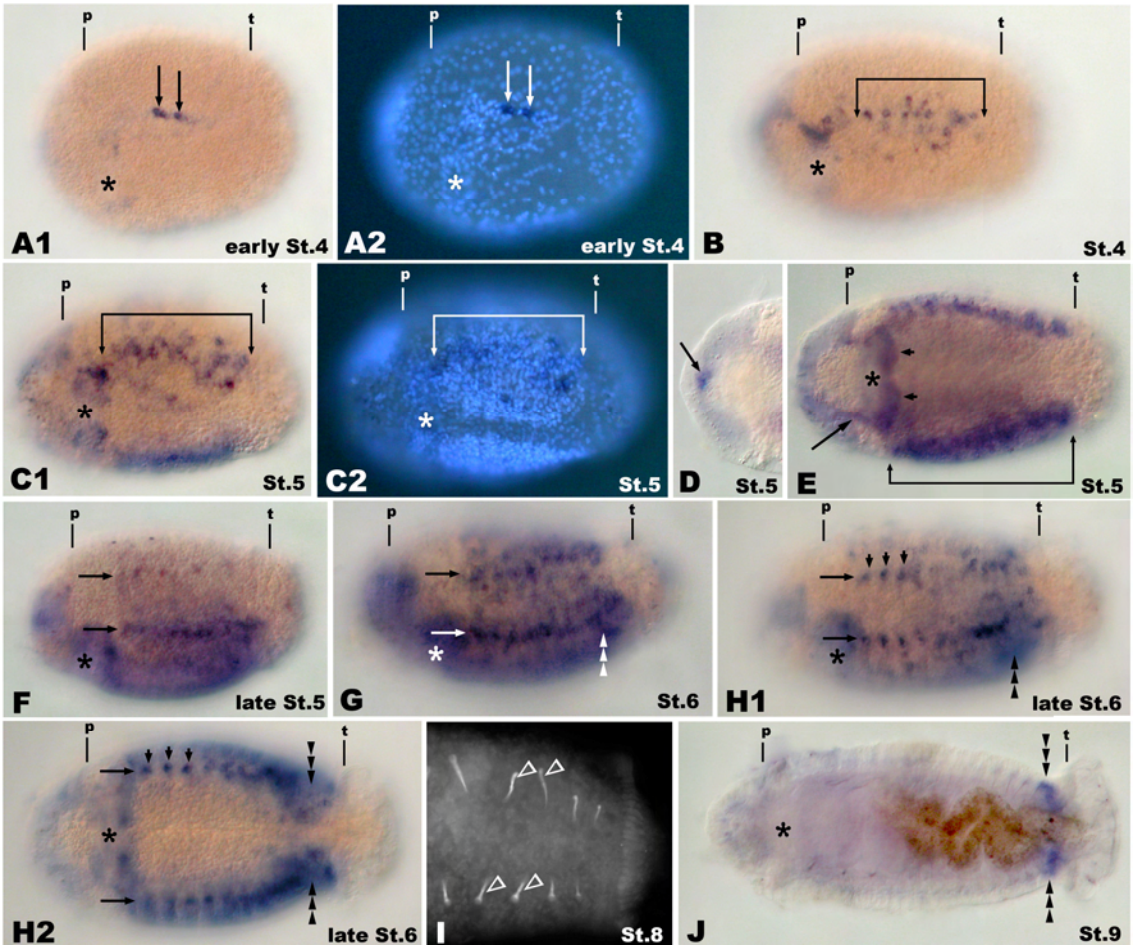


Fig. 8. *CapI-Delta* is broadly expressed in the developing segments and later in the presumptive chaetal sacs. A1, 2. Early St. 4; first signs of expression in the belly plates (arrows). **B.** St. 4; expansion of earlier expression in the presumptive segmented tissue (bracketed arrows). **C1, 2.** St. 5; location of the later expanded expression in the unsegmented and segmented part of the belly plates (bracketed arrows). **D.** St. 5; location of the transcript in the head (arrow). **E.** St. 5; uniform distribution of *CapI-Delta* along the bodyline (bracketed arrows); expression in the developing forgut (small arrows), in the brain and peristomial region (long arrow). **F.** Late St. 5; *CapI-Delta* in two rows of spots (arrows). **G.** St. 6; lateral expression of the two rows becomes stronger (arrows); upcoming higher concentration of transcript in the posterior ventral region of the larvae (arrowheads). **H1, 2.** Late St. 6; definition of individual spots (small arrows) in the two lateral rows of expression (arrows); higher concentration of *CapI-Delta* in the posterior (arrowheads). **I.** St. 8; arrangement of the chaetae in two lateral rows (open arrowheads). **J.** St. 9; restriction of expression to the ectodermal and mesodermal posterior region of the larvae (arrowheads). Anterior is to the left, dorsal up and ventral down for all pictures. A1, 2, B; C1, 2 are ventro-lateral views. F, G, H1 and I are lateral views. E, H2 and J are ventral views. A2 and C2 were exposed to Hoechst 33342. A1, 2, C1, 2 and H1, 2 are the same animals. Expression in St. 4 - late St. 6 is localized in the ectoderm. Asterisk marks the mouth. p-prototroch; t-telotroch.

posterior of the larvae is visible at stage 6 (Fig. 8G, arrowheads) and becomes stronger and more evident at late stage 6 (Fig. 8H1 and H2, arrowheads) together with the individual spots of expression in the chaetal sac anlagen.

Chaetogenesis is similar in all polychaetes and is initiated by the so called chaetoblast, which gives rise to specific microvilli within a funnel (Hausen 2005). In *Capitella* sp. I, two bilateral rows of chaetae, the noto- (dorsal) and neuro- (ventral) podial chaetae, are formed at around stage 7 (Fig. 8I, arrowheads). Just before the visible formation of the chaetae rows, *CapI-Delta* expression is being downregulated in the chaetal sac primordia (not shown). At later stages, *CapI-Delta* expression is restricted to the posterior growth zone of the larvae in the ectoderm and mesoderm (Fig. 8J), spanning two to three segments.

We can also detect expression of *CapI-Delta* in a region lateral and posterior to the mouth (Fig. 8E, arrow, small arrows) from stage 4 on. This expression domain remains through late stage 6 and could represent the circumesophageal nervous system. At early stage 5, an additional expression domain appears in the anterior midline of the head in the central nervous system of the embryo (Fig. 8D, arrow). This expression is still visible at stage late 6, but disappears around stage 7.

3.1.1 *Delta* in *Platynereis dumerilii*

An initial 596 bp fragment of *Pdu-Delta* was provided by the workgroup of Prof. Dr. H. Kress/ Berlin/ Germany. *Pdu-Delta* was RACEd out with the SMART-RACE kit to obtain the complete predicted ORF of 2445 bp, which translates into a 815 a.a predicted protein.

The conceptional translation of *Pdu-Delta* also shows the characteristics of a *Delta* protein already described for *CapI-Delta* in 3.1.1. The conserved DSL-domain is depicted in Fig. 7D aligned with the *CapI-Delta* sequence and other *Delta* proteins. The fact that the DSL regions are a modification of a normal EGF repeat is clearly visible. An EGF repeat is characterized by its cysteine residues and their arrangement. The general formula is $CX_4CX_5CX_8CXCX_8CX_6$, whereas the spacing between the last three cysteine residues is highly conserved (Wharton et al. 1985) and this can be seen in both *Delta* ORFs (Fig. 7b and c). The DSL region is typically longer in comparison to an EGF repeat and the spacing between the six identified cysteine residues is larger (Tax et al. 1994). Both including

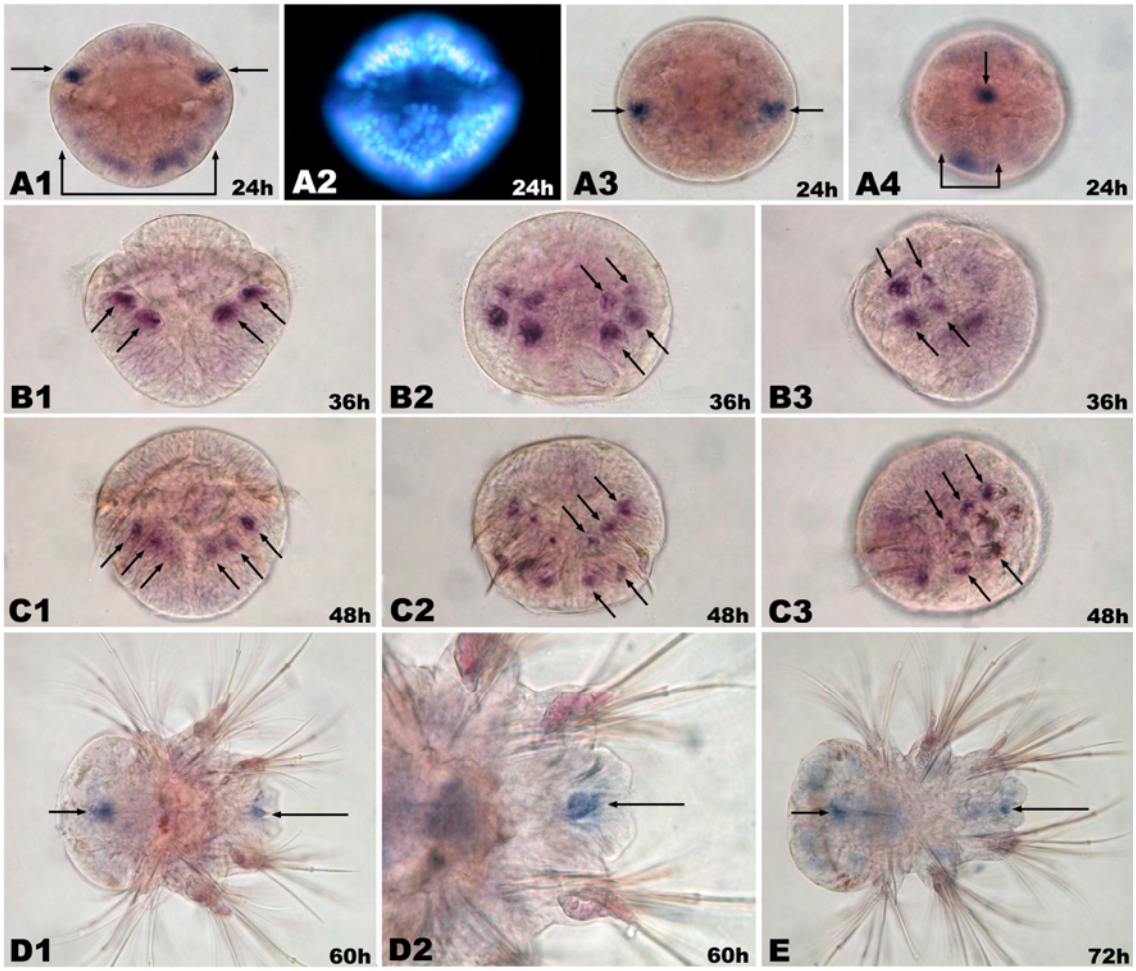


Fig. 9. *Pdu-Delta* is expressed in the presumptive chaetal sac anlagen and chaetal sacs and in the growth zone in later stages. A1, 2. 24h; *Pdu-Delta* expression in the region of the already developed larval eyes (arrows) and in the posterior of the animal (bracketed arrows) right next to the ventral plate (A2). **A3.** 24h; apical view shows symmetric bilateral expression. **A4.** 24h; expression is located above the prototroch (arrow) and posterior to the mouth (bracketed arrows). **B1.** 36h; *Pdu-Delta* expression in the future chaetal sacs (arrows). **B2.** 36h; *Pdu-Delta* is expressed in four bilateral chaetal sac anlagen (arrows). **B3**. 36h; anterior expression domains of the four patches on each side of the larvae are broader than posterior patches (arrows). **C1.** 48h; smaller expression domains in all three anterior chaetal sacs at each side of the larvae (arrows). **C2, 3.** 48h; localized bilateral expression of *Pdu-Delta* in five of six chaetal sacs (arrows). **D1.** 60h; anterior stomodaeum expression (small arrow) and transcript localization in the growth zone (long arrow). **D2.** 60h; close up of growth zone expression (long arrow). **E.** 72h; sustained but weaker stomodaeum (small arrow) and growth zone expression (long arrow). 24-48h: anterior is up, ventral down and dorsal up. 60h-3W: anterior is to the left, ventral down and dorsal up. A1, 2, B1, C1, D1, E2 and E are ventral views. A3 is an apical view. A4, B3 and C3 are lateral views. B2 and C2 are ventro-posterior views. A2 was exposed to Hoechst 33342. A1-4, B1-3, C1-3 and D1, 2 are the same animals. C1 is a merge of two foci of the same animal.

CapI-Delta and *Pdu-Delta* sequences show these features. The *Pdu-Delta* sequence also contains nine EGF repeats comparable to *CapI-Delta*, a signal signal sequence, the transmembrane region and the intracellular domain (Fig. 7c).

The expression pattern of *Pdu-Delta* over time is very specific and restricted. At 24h, prominent expression in the presumptive larval eye patches can be detected (Fig. 9A1, arrows) anterior to the prototroch (Fig. 9A4, arrow). The exact bilateral location of the expression can be seen from an apical view (Fig. 9A3, arrows). *Pdu-Delta* is also expressed posterior to the mouth, but is very faint (Fig. 9A1 and A4, bracketed arrows) at the border of the ectoderm and mesoderm of the embryo in patches, which might represent the future chaetoblasts.

According to Wilson (Wilson 1892), *P. dumerilii* forms two first 'somites' between 24h and 40h of development. The third somite forms later after 40h. At the same time, the first two chaetal pairs appear at each side of the animal. Later on the third pair of chaetae on both sides of the larva is formed.

At 36h, *Pdu-Delta* is expressed in four of the six chaetal sac anlagen at each side of the embryo (Fig. 9B1-3 arrows). The two most ventral located anlagen show a higher level of *Pdu-Delta* expression than the dorsal patches of expression (Fig. 9B2 and 3, arrows). In comparison to the dorsal expression domains, approximately three to four additional cells express *Pdu-Delta* in the ventral patches of the presumptive chaetal sacs. The expression coincides with the described formation of somites and the appearance of chaetae after Wilson (see above).

Later on at around 48h of development, the patches of expression become smaller, but *Pdu-Delta* is now expressed in five of the six already formed chaetal sacs (Fig. 9C2 and 3, arrows). The chaetae have protruded the surface of the embryo (Fig. 9C1) and only the most dorsal lowest chaetal sac does not show signs of *Pdu-Delta* expression (Fig. 9C3).

After the formation of three pairs of chaetae on each side of the larvae, the expression shifts towards the posterior growth zone (Fig. 9D1 and 2, long arrow) and the stomodaeum (Fig. 9D1, small arrow). This expression pattern remains through 72h of development (Fig. 9E, small and long arrow). We can not exclude, that the described expression in the posterior of the larvae is located in the hindgut. Later expression of *Pdu-Delta* could not be detected (5 and 7 d: 2, 3 and 4 weeks of development).

3.2 The receptor *Notch*

3.2.1 *Notch* in *Capitella* sp. I

The following primers were used in a seminested PCR with SMART RACE cDNA as template to isolate a 89 bp putative *Notch* fragment: anknotchFout, CDC3.2 and anknotchF1in. The resulting fragment corresponded to the fourth ankyrin repeat of the *Notch* intracellular domain (NICD) (see Fig. 10a and b). By performing RACE PCR, we were able to obtain a 2321 bp fragment yielding a 1682 bp predicted ORF with 561 deduced a.a.. Besides the 4th ankyrin repeat, the sequence also possesses a 5th and 6th ankyrin (also called CDC-10) repeat, a putative PEST domain and an opa-repeat (Fig. 10b). The presence of these domains, characteristic for typical *Notch* proteins (Coffman et al. 1990; Ellisen et al. 1991; Reaume et al. 1992; Wharton et al. 1985; Yochem and Greenwald 1989), identifies our isolated fragment as a *Notch* homologue, which we called *CapI-Notch.* The RAM23 domain, also part of the NICD in *Notch* proteins and evidently indispensable for the binding of *Notch* to the transcriptional repressor Su(H) or RBP-Jκ (Tamura et al. 1995), is missing in the *CapI-Notch* sequence as well as the first three ankyrin repeats.

Expression of *CapI-Notch* was analysed using a 2.2 kb fragment of the putative *Notch* fragment. Initial expression of *CapI-Notch* was detected in stage 4 (Fig. 11A1 and 2, arrow) in the developing belly plates. The expression in the presumptive segmented tissue expands to the posterior and becomes broader (Fig. 11B1 and 2, bracketed arrows). At stage 5, the expansion of expression is mainly limited towards the dorsal side of the embryo (Fig. 11D, bracketed arrows). At late stage 6, this broad lateral expression in the belly plates becomes very refined into a two row expression pattern (Fig. 11F2, arrows) comparable to but not exactly the same as the one observed in *CapI-Delta*. *CapI-Notch* is expressed in the presumptive chaetal sacs as segmentally arranged patches (Fig. 11F2, small arrows) in the mentioned two row pattern. The expression resolves right before the formation of the chaetae at stage 7 and disappears(Fig. 11G).

At stage 6, a ventral view shows the lateral expression of *CapI-Notch*, which is slightly stronger at the anterior of the larvae (Fig. 11E, bracketed arrows). A few hours later, this expression shifts towards the posterior segments also visible in a lateral view

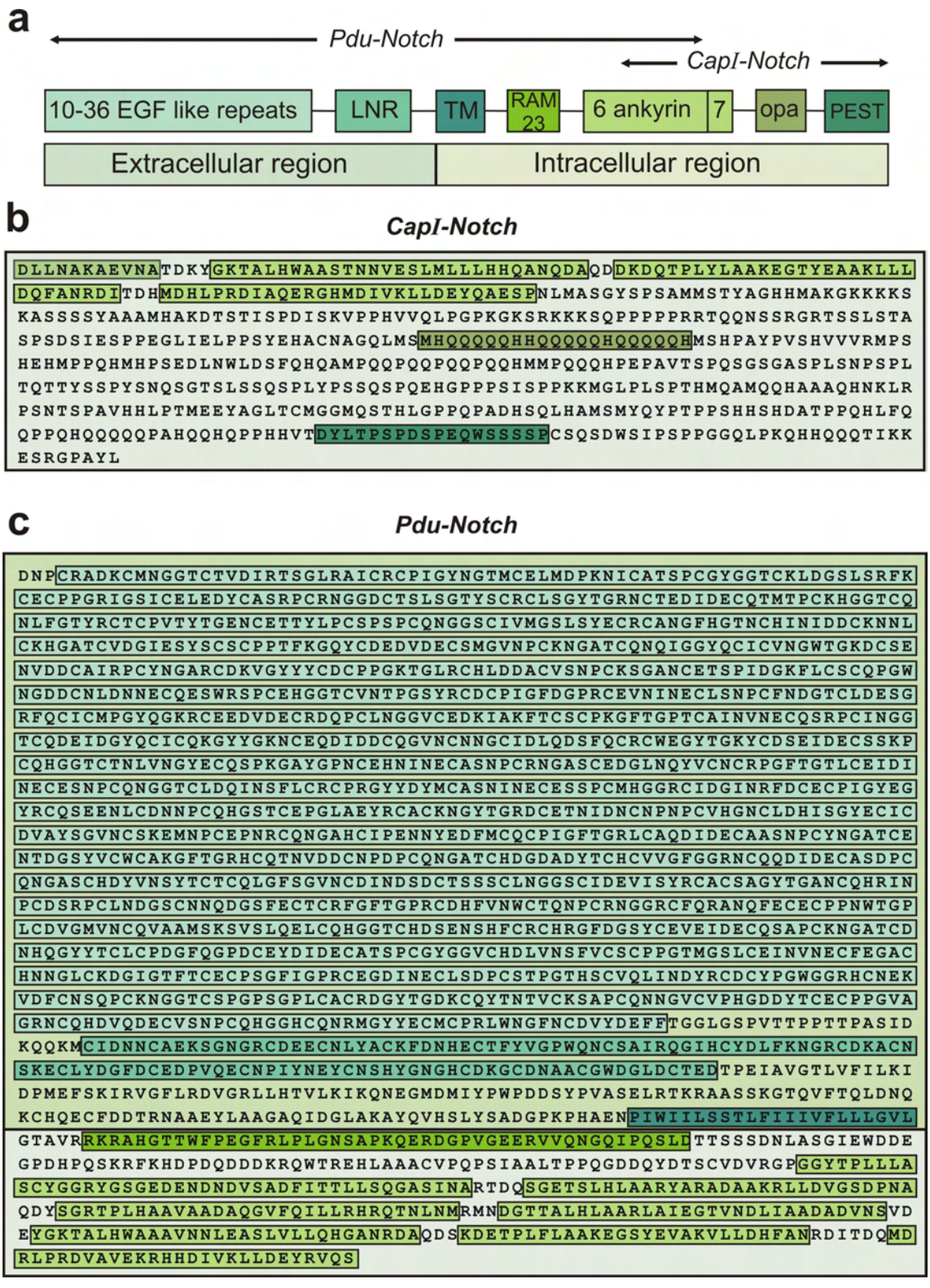


Fig. 10a. General arrangement of the conserved regions in a *Notch*-protein and location of the obtained ORFs of *CapI-Notch* and *Pdu-Notch*. LNR - lin12-repeats; TM – transmembrane region; RAM23 – RAM23-domain; 7 – 7th putative ankyrin repeat; opa – opa-domain; PEST - PEST-region. **10b. Isolated 3`-RACE 2.2 kb fragment of *CapI-Notch*.** Colours of the labeled regions correspond to the colours in 10a. **10c. *Pdu-Notch* sequence** (obtained from Genebank). Colours of the labeled regions correspond to the colours in 10a.

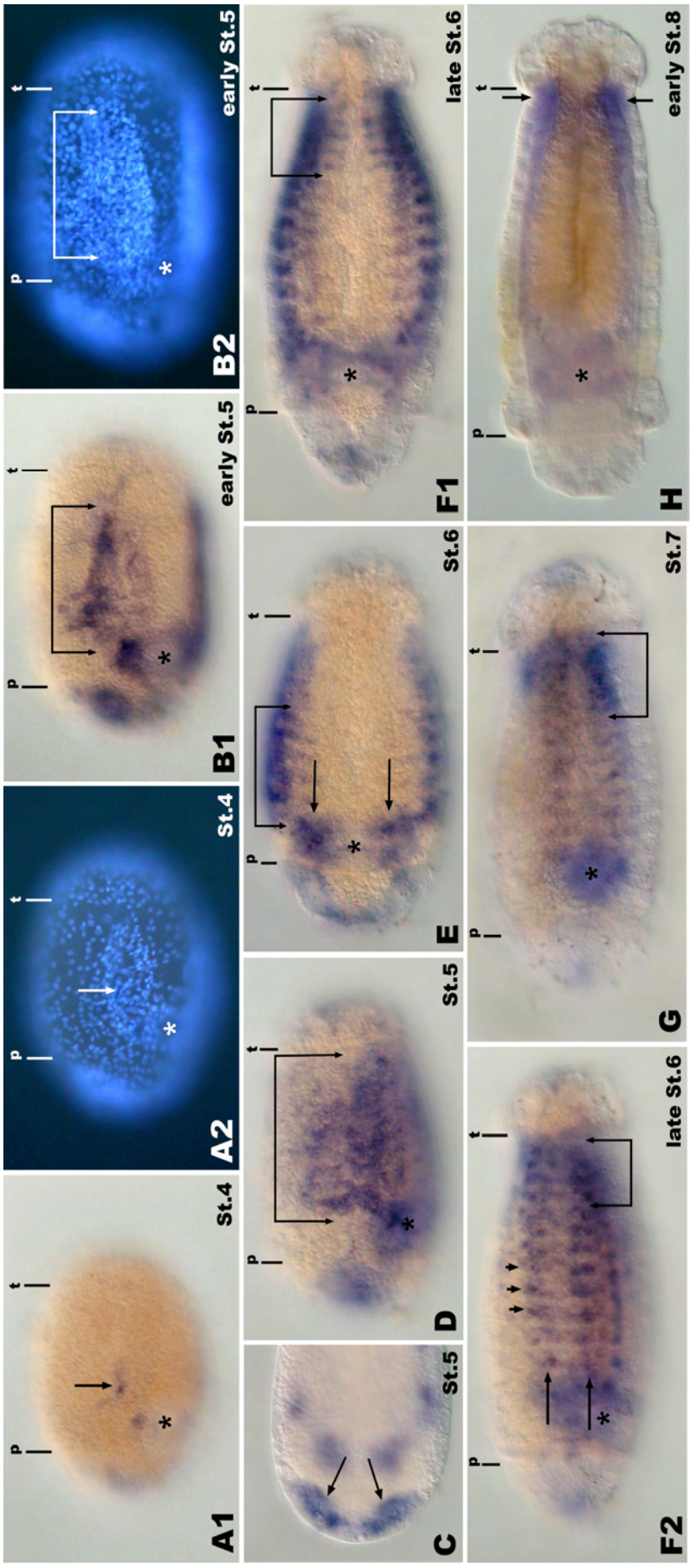


Fig. 11. *CapI-Notch* is broadly expressed during larval segment formation. A1, 2. St. 4; initial *CapI-Notch* expression in the belly plates (arrow). **B1, 2.** Early St. 5; expansion of expression in segmented and unsegmented region of the belly plates (bracketed arrows). **C.** St. 5; expression in the developing brain (arrow). **D.** St. 5; dorsal expansion of *CapI-Notch* expression and uniform transcript distribution from anterior to posterior (bracketed arrows). **E.** St. 6; more intense expression in the anterior of the larva (bracketed arrow) and in the developing foregut (arrows). **F1.** Late St. 6; higher expression level at the posterior of the larvae and extension to the ventral midline (bracketed arrows). **F2.** Late St. 6; maturation of the uniform lateral expression into a segmental (small arrows) two-row (long arrows) expression; prominent posterior *CapI-Notch* expression (bracketed arrows). **G.** St. 7; localized expression in the posterior segments (bracketed arrows). **H.** Early St. 8; restricted expression in the posterior growth zone (arrows). Anterior is to the left, dorsal up and ventral down for all pictures. A1, 2 and B1, 2 are ventro-lateral views. D, F2, G are lateral views. C, E, F, I, H are ventral views. A2 and B2 were exposed to Hoechst 33342. A1, 2, B1, 2 and F1, 2 are the same animals. Asterisk marks the mouth. p-prototroch; t-telotroch.

and 2, bracketed arrows). At stage 7, *CapI-Notch* expression has almost disappeared on either side of the larvae, but it remains in the posterior-most four to five segments (Fig. 11G, bracketed arrows). Expression domains can also be detected at early stage 8, yet is exclusively in the ecto-and mesoderm of the most posterior two to three segments (Fig. 11H, arrows). *CapI-Notch* is also expressed bilaterally next to the mouth (Fig. 11E, arrows) beginning at late stage 4 to early stage 5, remaining through stage 7 and fading out around late stage 7 to early stage 8. Prominent expression in both lobes of the brain occurs at early stage 5 (Fig. 11C, arrows). The central nervous system expression of *CapI-Notch* remains until late stage 6 and is no longer detectable anymore around stage 7.

3.2.2 *Notch* in *P. dumerilii*

The *Pdu-Notch* sequence was retrieved from the NCBI-blast databank (CAJ38792). Specific primers were designed to amplify a 515 bp *Pdu-Notch* fragment from mixed stage cDNA. The product was introduced into a vector and cloned. To verify the direction and the *Pdu-Notch* gene in the plasmid, several plasmids were sequenced. The primers correspond to the 5th EGF repeat and to the 9th EGF repeat in the extracellular domain of *Pdu-Notch.*

The published *Pdu-Notch* sequence possesses many characteristic and conserved domains, also present in *Notch* genes of other organisms (Fig. 10a and c). It has an extracellular (ECD) and intracellular domain (ICD). The ECD usually consists of 35 EGFs, in which the 11th and 15th repeat only possesses five instead of six cysteine residues. All other EGFs show a high similarity to the general formula for these repeats (see 3.1.1).

Pdu-Notch also possesses a transmembrane region, the RAM23 domain and 6 ankyrin repeats as well as the 7th putative ankyrin repeat (Fig. 10a and c). Evidently, the published *Pdu-Notch* sequence is not complete and parts of the 3' ORF are missing, which should contain a PEST-domain as well as an opa-repeat (compare to 3.2.1).

The cloned 515 bp fragment of *Pdu-Notch* was used to generate a probe for in-situ hybridization. At 24h, *Pdu-Notch* is expressed almost uniformly in the episphere and the hyposphere of the embryo (Fig. 12A1, bracketed arrows). A vague demarcation of the stomodaeum expression can be seen in the ventral and posterior view (Fig. 12A1 and 3, arrow). The dorsal side of the embryo also shows uniform episphere and hyposphere

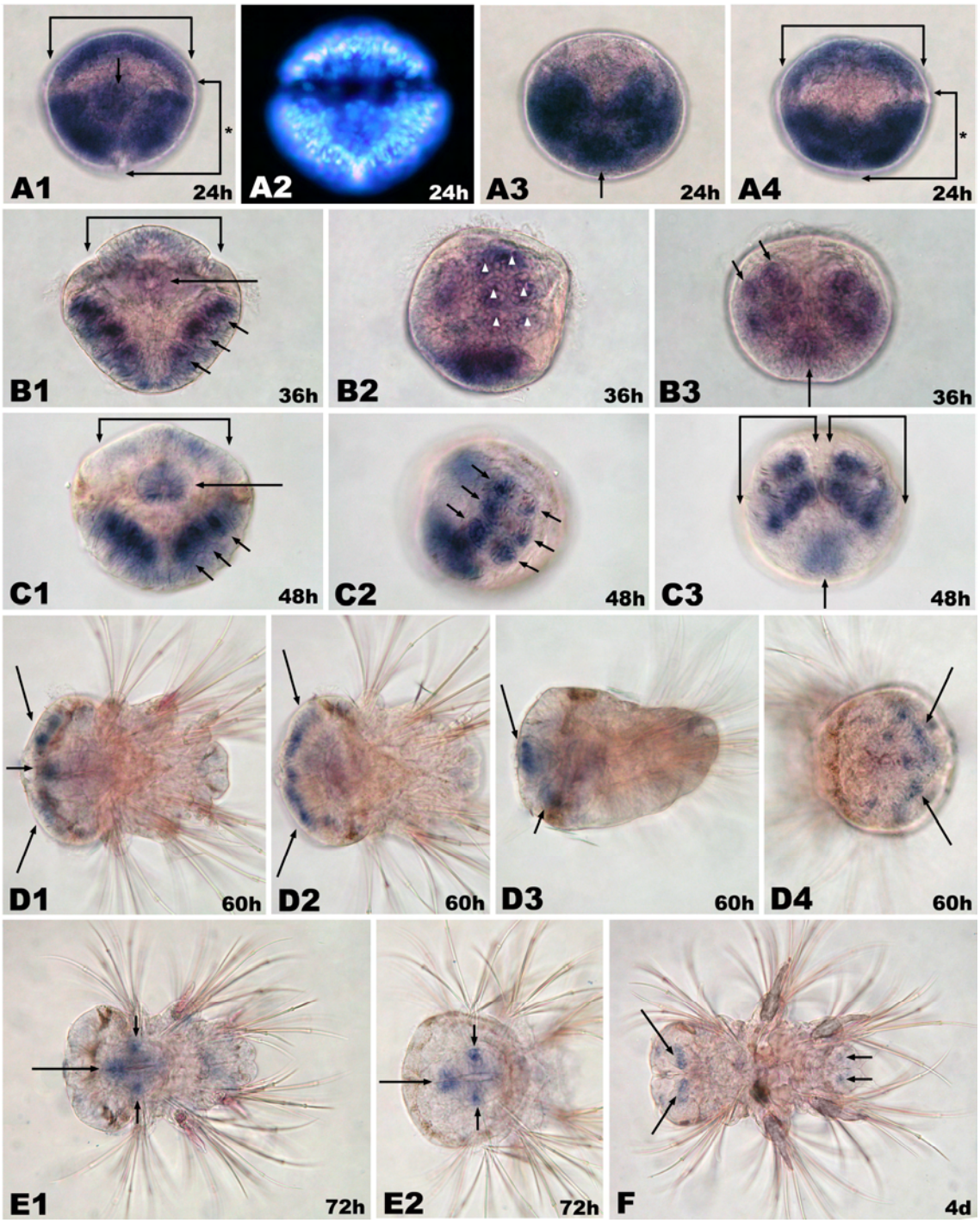


Fig. 12. *Pdu-Notch* is expressed in the chaetal sacs and the central as well as in the stomatogastric nervous system. A1-4. 24h; broad expression of *Pdu-Notch* in the episphere (bracketed arrows) and hyposphere (asterisk bracketed arrows) of the embryo; stomodaeum expression is labeled separately (arrow). **B1.** 36h; mouth expression (arrow) as well as brain expression (bracketed arrow) become weaker, but more specific; hyposphere expression is now mostly limited to the developing chaetal sacs (small arrows). **B2.** 36h; lateral view shows three pairs of developing chaetae with *Pdu-Notch* expression (arrowheads). **B3.** 36h; broad expression around and in the chaetae is visible (arrows). **C1, 2.** 48h; central nervous system (bracketed arrows) as well as mouth (long arrows) expression sustain; chaetae expression becomes more localized (small arrows). **C3.** 48h; bracketed arrows show limited expression of *Pdu-Notch* in the three pairs of chaetal sacs on both sides of the larva; arrow = mouth expression. **D1-4.** 60h; expression in the cerebral ganglia (long arrows) and mouth (small arrows). **E1, 2.** 72h; limited expression in the anterior (long arrows) and lateral region of the stomodaeum (small arrows). **F.** 4d; shift of expression to the predicted palp anlagen (long

arrows); faint expression of *Pdu-Notch* in the growth zone (small arrows). 24-48h: Anterior is up, ventral down and dorsal up. 60h-3W: Anterior is to the left, ventral down and dorsal up. A1, 2, B1, C1, D1 and E1 are ventral views. A3, B3 and C3 are ventro-posterior views. A4, D2 and F are dorsal views. B2, D3 and C2 are lateral views. D4 as well as E2 are ventro-apical views. A2 was exposed to Hoechst 33342. A1-4, B1-3, C1-3, D1-4 and E1, 2 are the same animals. C1 is a merge of two foci of the same animal.

expression domains with the exception of the region around the prototroch (Fig. 12A4, bracketed arrows).

Twelve hours later, the uniform expression of *Pdu-Notch* becomes more limited to certain regions of the embryo. Three expression domains can be distinguished, which are located in the central nervous nervous system (Fig. 12B1, bracketed arrows), the stomodaeum (Fig. 12B1 and 2, long arrow) and the presumptive chaetal sac anlagen (Fig. 12B1-3, small arrows and arrowheads). The expression in the chaetal sacs is very broad, but located in the ectoderm. All six future chaetal sacs on each side of the embryo express *Pdu-Notch* (Fig. 12B2, arrowheads).

The expression in the central nervous system as well as in the stomodaeum persists through 48h of development (Fig. 12C1, bracketed arrows, long arrow). *Pdu-Notch* is also still expressed in the chaetal sacs, which have already formed by now, including the chaetae themselves. The expression becomes even more refined and is stronger in the more ventral row of chaetae in comparison to the dorsal row (Fig. 12C2, small arrows). Fig. C3 shows very limited expression of *Pdu-Notch* in and around the three pairs of chaetae on each side of the embryo (bracketed arrows).

The prominent chaetal sac expression disappears completely at 60h of development, but the expression domains of the stomodaeum as well as of the central nervous system remain and become even more distinct (Fig. 12D1, small arrow and long arrow). The expression of the mouth is located at the anterior of the stomodaeum as a single patch (Fig. 12D3, small arrow). The *Pdu-Notch* expression in the central nervous system spans the most anterior of the head in a sickle shape. The band of expression is approximatley one to two cells wide in the ventral-dorsal dimension (Fig. 12D2, arrows) and is located right in the middle of the larvae from a lateral view (Fig. 12D3, long arrow).

At 72h, the expression in the central nervous system has also disappeared, but the stomodaeum expression becomes more complex. In addition to the anterior patch (Fig. 12E1, long arrow), two dots of expression bilateral to the stomodaeum appear (Fig. 12E1, small arrows). All three expression domains are located in the ectoderm (Fig. 12E2 close

up, arrows). The arrangement of the stomodaeum expression could mark the development of the stomatogastric nervous system or the foregut judging from its location

After another 24h of development, the expression of *Pdu-Notch* shifts to the presumptive palp anlagen (Fig. 12F, long arrows). Very weak expression is also detectable in the posterior growth zone in two distinct patches (Fig. 12F, short arrows). No expression could be detected in older stages (5 and 7d; 2, 3 or 4 weeks).

3.3 The target genes *hes*

3.3.1 Phylogeny of *hes* genes in *Capitella* sp. I and *P. dumerilii*

The identification of *hes / hairy* genes was more complicated than originally expected. There are several groups of genes in this family. The sequences contain a bHLH domain (basic helix-loop-helix) region, an Orange domain and a C-terminal tetrapeptide (Fig. 13). On the basis of the first two conserved regions, this group of genes is also called bHLH-O (basic helix-loop-helix-Orange) (Davis and Turner 2001). These proteins are involved in multiple mechanisms of development and bind to specific DNA regions recruiting transcriptional co-repressors (Davis and Turner 2001).

The group of bHLH-O genes contains four subgroups: the *hairy* genes, the *Enhancer of split* (*E(spl)*)genes, the *HESR/ hey* genes and the *Stra13/DEC* genes. This classification was determined by Davis and Turner in a phylogenetic analysis of all genes possessing bHLH and O conserved regions.

The *hairy* and *hes* genes possess a proline residue right at the beginning of the bHLH region (Fig. 13), whereas the *hey* genes have a glycine residue at that position. We had to determine what genes of the bHLH-O family we had actually isolated. First, the sequences were aligned and compared by eye to other proteins of the four different groups of the family. The conservation of certain amino acids in the three conserved domains already revealed information on the isolated sequences (Fig. 13).

Next, different phylogenetic analyses were performed with the available sequences of *Capitella sp. I* and *P. dumerilli* and a consensus tree was created containing the credibility values of those three different analyses (Fig. 14). All names of the proposed bHLH-O genes in *Capitella* sp. I and *P. dumerilii* genes were given after the phylogenetic analysis results.

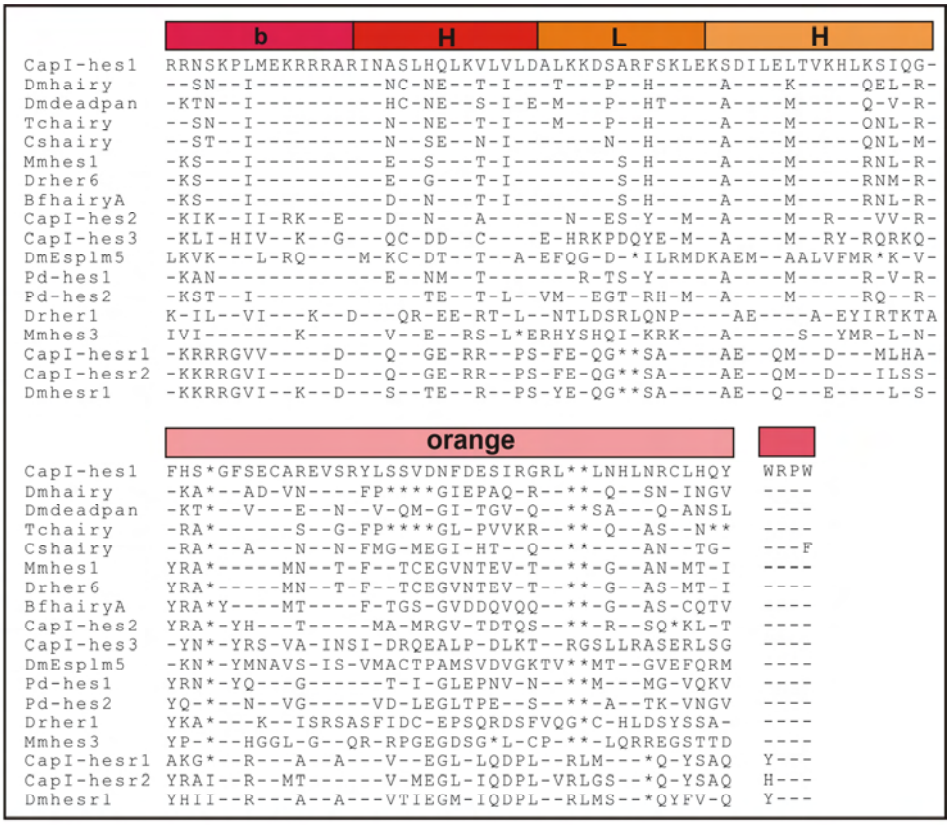


Fig. 13. Alignment of the conserved bHLH-domain (basic-Helix-Loop-Helix), Orange domain and WRPW-tetrapeptide. Dashes indicate identical amino acids.* marks gaps. Dm-*Drosophila melanogaster*; Tc-*Tribolium castaneum*; Cs-*Cupiennius salei*; Dr-*Danio rerio*; Mm-*Mus musculus*; Bf-*Branchiostoma floridae*;

Five *hes / hairy* genes of *Capitella* sp. I and two sequences of *P. dumerilii* were isolated and used for phylogenic analyses. *CapI-hes1* was isolated by performing degenerate PCR (see 3.3.2). *CapI-hes2* and *CapI-hes3* as well as *CapI-hesr1* and *CapI-hesr2* were found by searching through the trace files of the sequenced *Capitella* sp. I genome (Joint Genome Institute, Walnut Creek, CA, USA). The *hes*-sequences of *P. dumerilii*, *Pdu-hes1* and *Pdu-hes2,* were also isolated by performing degenerate PCR (see 3.3.3 and 3.3.4).

All three analyses show that *CapI-hesr1* and *Cap-hesr2* belong to the *hesr* and *hey* gene group (Fig. 14). Both genes also have characteristic amino acids in their sequence, adding additional support to this assignment, like the glycine residue in the beginning of the bHLH-region instead of the proline like in *hairy* or *Enhancer of split* genes (see above).

CapI-hes2 and *Cap-hes3* belong to the *E(spl)* family and group together with the *Enhancer of split* genes of *Drosophila.* The invertebrate *E(spl)* group has *Pdu-hes1* at its base, which would also place it into the *E(spl)* gene group rather than into the *hairy* gene family. The

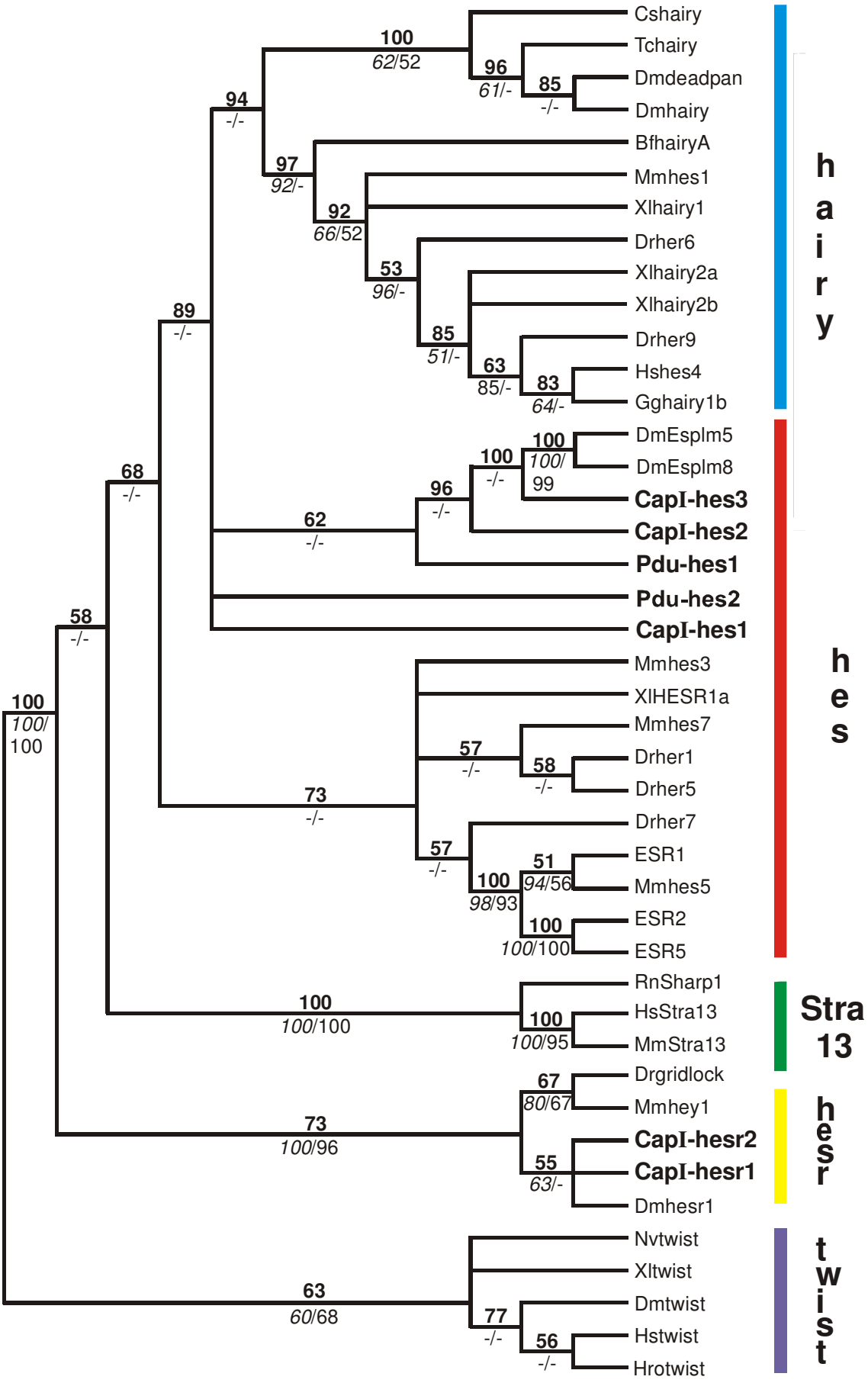

Cshairy
Tchairy
Dmdeadpan
Dmhairy
BfhairyA
Mmhes1
Xlhairy1
Drher6
Xlhairy2a
Xlhairy2b
Drher9
Hshes4
Gghairy1b
DmEsplm5
DmEsplm8
CapI-hes3
CapI-hes2
Pdu-hes1
Pdu-hes2
CapI-hes1
Mmhes3
XlHESR1a
Mmhes7
Drher1
Drher5
Drher7
ESR1
Mmhes5
ESR2
ESR5
RnSharp1
HsStra13
MmStra13
Drgridlock
Mmhey1
CapI-hesr2
CapI-hesr1
Dmhesr1
Nvtwist
Xltwist
Dmtwist
Hstwist
Hrotwist
hairy
hes
Stra 13
hesr
twist

Fig. 14. Phylogenetic analysis of *Capitella* sp. I and *P.dumerliii* bHLH-O genes. The alignment for the tree was generated from 43 bHLH-O-genes. The bayesian tree is shown with its confidence values in bold above the branches. A majority tree was generated from 9500 different trees. The neighbor joining analysis (NJ) is being represented by the values under the branches in italic. A 1000 bootstrap repetitions were performed for the NJ-analysis as well as for the Parsimony analysis, which is represented by the values under the branches right hand of the NJ-values. The five *twist* genes were used as an outgroup, which also contains a bHLH-domain. Hairy-*hairy* genes, hes- *Enhancer of split* genes; *hesr-enhancer of split related* genes and *hey* genes, *Stra13-Stra13/DEC/SHARP* gene family, Twist- *twist* genes; '**-** -' values below 50. Gene abbreviations: CapI- *Capitella* sp. I; Pd-*Platynereis dumerilii;* Espl-*enhancer of split* genes; hes-*hairy* and *Enhancer of split* genes; her-*hairy* and *enhancer of split* related genes; Dm-*Drosophila melanogaster;* Tc-*Tribolium castaneum;* Cs-*Cupiennius salei;* Mm-*Mus musculus;* Dr-*Danio rerio;* Gg-*Gallus gallus;* Bf-*Branchiostoma floridae;* Hro-*Helobdella robusta*; Xl-*Xenopus laevis*; Nv-*Nematostella vectensis*; Ce-*Caenorhabditis elegans*; Hs-*Homo sapiens*;

assignment of the *Pdu-hes2* and *CapI-hes1* to one of the four bHLH-O groups is not so evident and well supported as the placement of the other five genes.Both genes are placed at the base of the invertebrate *E(spl)* genes and *hairy* genes and not into the separate family of the vertebrate *E(spl)* genes. A better resolution of the Bayesian consensus tree was not possible. The Parsimony and Neighbour-Joining analyze resulted in trees with significant polytomies. The NJ analysis places *CapI-hes1* and *CapI-hes, Pdu-hes1,* and *Pdu-hes2* into the *hairy* family.

3.3.2 *CapI-hes1*

With the primers HES-leech-fw (Song et al. 2004), h-bw1 and h-bw2 (Damen et al. 2000) and a semi-nested PCR, a 112 bp putative *hes* fragment was isolated from a *Capitella* mixed stage cDNA library. By performing RACE PCR, a composite sequence of 872 bp was obtained containing a complete predicted ORF of 732 bp (244 a.a.). The sequence of the gene, which we call *CapI-hes1*, possesses many characteristics typical for the bHLH-O genes (Fig. 13). The bHLH region and the Orange domain are responsible for the repression of specific transcriptional activators (Dawson et al. 1995; Taelman et al. 2004). The C-terminal WRPW tetrapeptide displays a *groucho* interaction motif, which results in transcriptional repression by *hairy* (Fisher and Caudy 1998; Jimenez et al. 1997; Paroush et al. 1994). It also acts as a polyubiquitylation signal for the degradation of the Hes protein by the proteasome (Kang et al. 2005).

Phylogenetic analyses suggest the assignment of *CapI-hes1* to the invertebrate *E(spl)* group and *hairy* genes (see 3.3.2 and Fig. 14). In situ hybridization was done with a 534 bp 5`RACE fragment.

CapI-hes1 seems to be expressed in the mesoderm throughout the examined stages of development. A complementary staining with the nuclear marker Hoechst showed that *CapI-hes1* expression is not superficial but rather internally localized within the belly plates. A double in-situ hybridization with an ectodermal expressed gene (*Pax 3/7*) showed *CapI-hes1* expression in deeper layers of the tissue than *pax3/7* (Seaver and Kaneshige 2006). *CapI-hes1* also colocalizes with the mesodermal expressed gene *twist* in double in-situs (Dill et al. 2007)

CapI-hes1 expression is initiated around stage 4 in the presumptive segmental tissue (Fig. 15A1 and 2, arrows). At early stage 5, the expression in the belly plates has expanded rapidly over six to seven already formed and also future segments (Fig. 15B1, arrows). Two expression patterns can be distinguished at that time of development. First, a segmentally arranged striped like pattern of *CapI-hes1* expression in the unsegmented part of the anterior belly plates. The segments in this anterior region are already forming in the ventral lateral part of the presumptive tissue, but no signs of segmentation is visible where *CapI-hes1* expression is seen (Fig. 15B2 and 3, arrows). The second part of *CapI-hes1* expression is located in the not yet segmented posterior area of the belly plates as a patch-like expression pattern (Fig. B2 and 3, brackteted arrows). At a slightly older developmental stage (stage 5), *CapI-hes1* expression in the posterior belly plates also resolves into a segmental banded pattern, which precedes morphological segmentation (Fig. 15C, arrows). Fig. 15D1 and 2 show the stripes of *CapI-hes1* expression, which are approximately three to four cells wide along the anterior-posterior axis. Between the adjacent stripes, there are one to two cells, which do not express *CapI-hes1*.

At stage 6, the anterior expression domain gets weaker (Fig. 15E1 and 2), bracketed arrows), and the posterior expression is now more prominent and still in the unsegmented part of the belly plates (Fig. 15E1 and 2, asterisk bracketed arrows). At later stages (stage 7), *CapI-hes1* expression becomes limited to the mesodermal part of the one or two segments last formed and the posterior growth zone (Fig. 15F, arrows). Expression of *CapI-hes1* was also observed in the lateral region of the mouth (stage 5 to late stage 7), probably associated with the developing foregut. Because we observed consistent variability among individuals in this expression domain (either one, two or no patches of expression), we interpret this as either a dynamic expression pattern or transient expression in the region of the developing foregut.

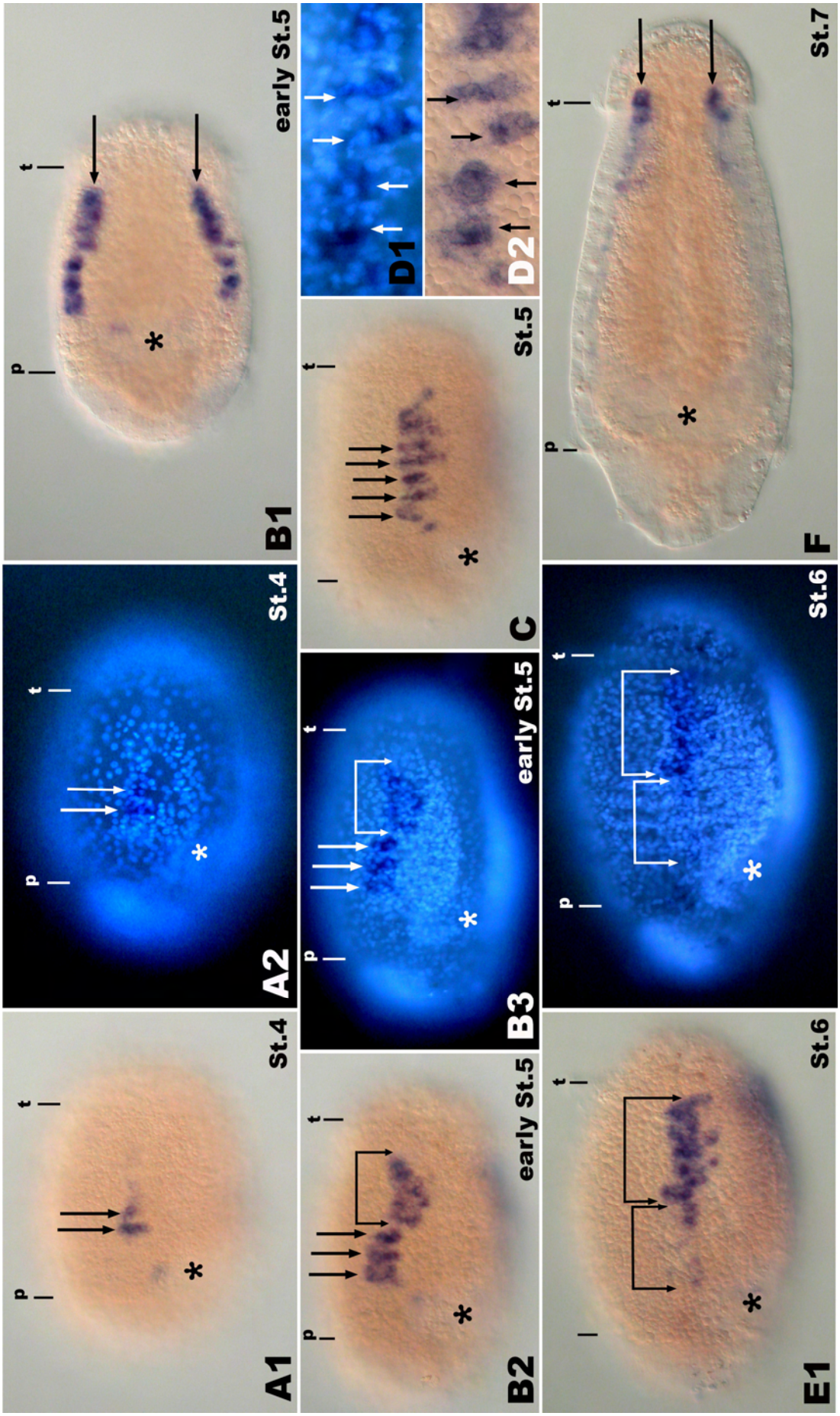

A1
St.4
A2
St.4
B1
early St.5
B2
early St.5
B3
early St.5
C
St.5
D1
D2
E1
St.6
St.6
F
St.7
t
p

Fig. 15. *CapI-hes1* is expressed before and during formation of larval segments in a segmental pattern. A1, 2. St. 4; first expression in the form of small patches in the presumptive segmented tissue (arrows). **B1.** Early St. 5; expansion of expression along the anterior-posterior axis (arrows). **B2, 3.** Early St. 5; anterior segmental expression pattern (arrows) and uniform posterior expression in the unsegmented part of the belly plates(bracketed arrows). **C.** St. 5; maturation of the expression into segmental stripes (arrows). **D1, 2.** Distinct bands of *CapI-hes1* (arrows). **E1, 2.** St. 6; downregulation of anterior expression (bracketed arrows), constant posterior *CapI-hes1* expression (asterisk and bracketed arrows). **F.** St. 7; limited expression in later stages in the mesodermal posterior growth zone (arrows). Anterior is to the left, dorsal up and ventral down for all pictures. A1, 2, B2, 3, C, E1, 2 are ventro-lateral views. B1, F are ventral views. A2, B3, D1 and E2 were exposed to Hoechst 33342. A1, 2, B1-3, D1, 2 and E1, 2 are the same animals. Asterisk marks the mouth. p-prototroch; t-telotroch.

3.3.3 *CapI-hes2* and *CapI-hes3*

CapI-hes2 and *CapI-hes3* were both found by searching through the trace files of the *Capitella* sp. I genome (see 3.3.1). The expression patterns of both genes in larvae and juveniles (see 3.9) were studied by Elaine C. Seaver (unpublished results). A short description of their larval expression relevant for the discussion of the results will follow (see 4.).

CapI-hes2 and *CapI-hes3* expression appears prior to segment formation in the developing belly plates, though the *CapI-hes3* transcript can be detected earlier (St. 4) than *CapI-hes2* (St.5) (Fig. 16A; Fig. 17A1, 2). Expression of both genes includes segmented and unsegmented portions of the presumptive segmented tissue and expands circumferentially following the expansion of the belly plates (Fig. 16B, C1, 2; Fig. 17B, C1, 2). At St. 7, *CapI-hes2* and *CapI-hes3* expression can be detected prominently in the mesoderm and weaker in the ectoderm of the segmented part of the larval body (Fig. 16E1, 2; Fig. 17E1, 2). Expression patterns of both genes become more refined towards the posterior of the larvae including the newly formed segments and the posterior growth zone (Fig. 16D, E1, E2; Fig. 17E, F2). Later on at St.9, *CapI-hes2* and *CapI-hes3* expression becomes restricted to the mesoderm and to a lesser extend to the ectoderm of the posterior growth zone (Fig. 16F; Fig. 17G).

At St. 5, *CapI-hes2* expression can be detected specifically in the future dorsal and ventral row of the developing chaetae expressed as discrete patches (Fig. 16B, dr and vr), This expression becomes even more pronounced at St. 6 (Fig. 16D). The segmentally iterated expression in the chaetal sac primordia ceases immediately before the formation of chaetae at St. 7 (Fig. 16E1). This refined chaetal sac expression could not be detected for *CapI-hes3*.

Both genes, *CapI-hes2* and *CapI-hes3*, are also expressed in the developing larval brain, the foregut and the hindgut at different developmental stages (Fig. 16A-E2 br, fg, hg; Fig. 17A-F1 br, fg; hg not shown).

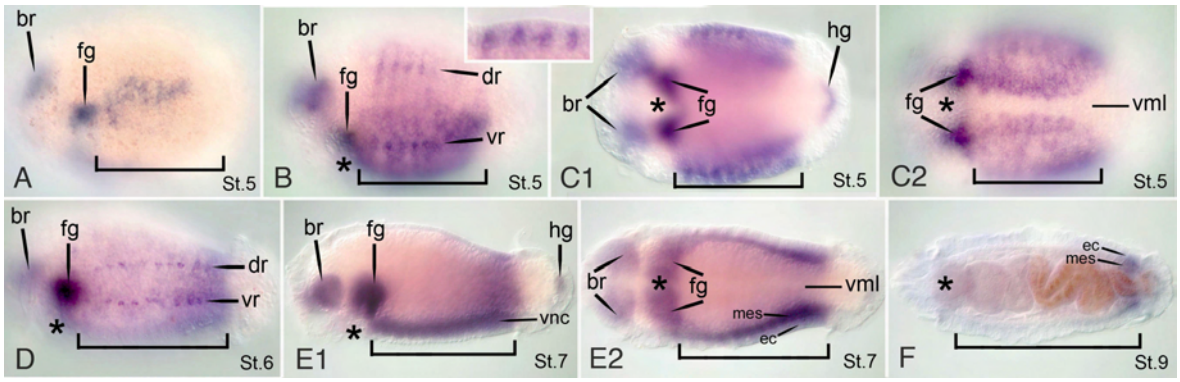


Fig. 16. Larval expression of *CapI-hes2*. A. Early St. 5; initial larval expression in the brain (br), foregut (fg) and presumptive segmental tissue (bracket). **B**. Late St. 5; *CapI-hes2* expression throughout the nascent and presumptive segments, and in both the dorsal (dr) and ventral (vr) presumptive chaetal sacs of the nascent segments. Inset shows a closeup of the expression in the dorsal row of presumptive chaetal sacs. **C1, 2.** St. 5; *CapI-hes2* transcript in the brain, foregut, hindgut and the segmental tissue (bracket). **D.** St. 6; *CapI-hes2* refined expression in both, the dorsal and ventral segmentally arranged presumptive chaetal sacs. **E1, 2.** St. 7; expression in the ventral nerve cord (vnc), in the segmental mesoderm (mes) and weaker expression in the segmental ectoderm (ec). **F.** *CapI-hes2* expression the ectoderm and mesoderm of the posterior growth zone. Anterior is to the left, dorsal up and ventral down for all pictures. Asterisk marks the mouth. vml, ventral midline. A is a ventro-lateral view; B, D, E1 are lateral views. C1, 2, E2 and F are ventral views. C1, 2 and E1, 2 are the same animals.

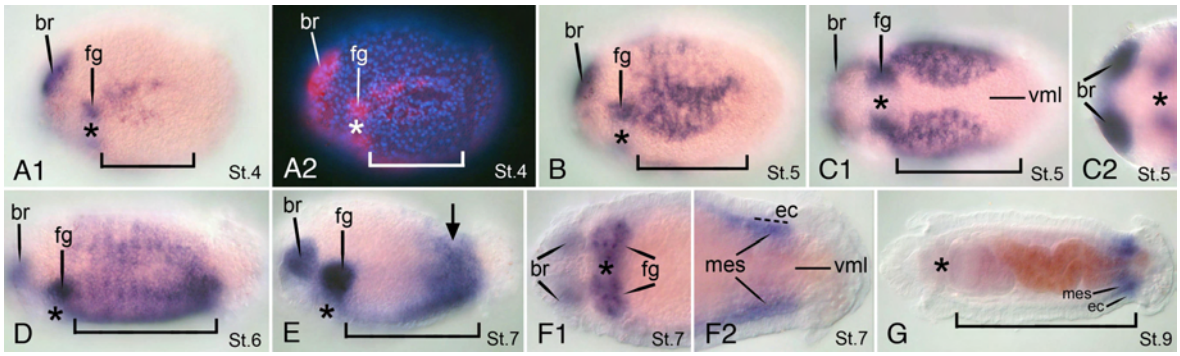


Fig. 17. *CapI-hes3* is broadly expressed during larval segment formation. A1, 2. St. 4; *CapI-hes3* expression in the brain (br), foregut (fg) and in a small ventro-lateral domain in the presumptive segmental tissue (bracket). **B, C1.** St. 5; broad expression of *CapI-hes3* in the presumptive segmental tissue (bracket). **C2.** St. 5; *CapI-hes3* transcript in both hemispheres of the developing brain placodes (br). **D.** St. 6; expansion of expression throughout the segments (bracket). **E, F2.** St. 7; expression in the anterior segments diminishes, posterior mesodermal (mes) expression remains (arrow). **F1.** St. 7; prominent expression in the foregut. **G.** St. 9; *CapI-hes3* transcript restricted to the ectoderm and mesoderm of the posterior growth zone. Anterior is to the left, dorsal up and ventral down for all pictures. Asterisk marks the mouth; ec, ectoderm; vml, ventral midline. Brackets mark the segmented (or presumptive segmented) region of the body. A1, 2 and B are ventro-lateral views. D and E are lateral views. C1, 2, F1, 2 and G are ventral views. A1, 2, C1, 2 and F1, 2 are the same animals. A2: Hoechst 33342 is in blue and the reaction product marking the *CapI-hes3* transcript is false-colored in red.

3.3.4 *Pdu-hes1*

The primers Csh-fw1, h-bw1 and h-bw2 (Damen et al. 2000) and mixed stage cDNA were used to perform a PCR and isolate a putative 138 bp fragment of a *hes*-gene. A complete predicted ORF of 1011 bp and translated sequence of 337 amino acids was obtained with 5` and 3`RACE PCR. All clones sequenced from the 5'RACE were identical. The 3`RACE resulted in two different sequences, both confirmed by three and four individual clones each. The composite ORF of all seven clones (nucleic acid and amino acid) was identical, but the 3' UTR differed in three sequence stretches 35, 10 and 40 nucleic acids in length. Due to this discrepancy, two composite sequences of 2358 bp and 2362 bp were isolated. Two different spliced products could be one reason for the differences in the UTRs, but the heterogeny of the *Platynereis dumerilii* gene pool might be another explanation.

The resulting ORF also has all characteristics of a *hes / hairy* gene. The sequence contains a bHLH domain, an Orange region and the WRPW motif (see 3.2.1 and 3.2.2) (Fig. 13). The *P. dumerilii* gene, which we called *Pdu-hes1*, is placed on the base of the invertebrate *E(spl)*-group in the phylogenetic analysis (Fig. 14). In-situs analysis was performed with a 5' RACE product to avoid uninterpretable results with a probe containing the variable 3' UTR regions of *Pdu-hes1*. At 24h of development, *Pdu-hes1* expression is very broad in both the episphere including the central nervous system and the hyposphere of the embryo (Fig. 18A1-A4, bracketed arrows and asterisk bracketed arrows A3 and 4). There is a gap of expression in the region of the prototroch on the ventral side of the embryo. Distinct mouth expression is detectable (Fig. 18A1 and 2, arrow). The posterior view shows no signs of expression at the future anus (Fig. 18A2). The epispheric expression does not go all the way around the embryo and shows no expression right in the middle of the dorsal more apical side in addition to the lack of expression in the region of the prototroch (Fig. 18A3). A different embryo at the same stage of development shows the stomodaeum expression more specifically (Fig. 18B, arrow).

The expression in the central nervous system and around the stomodaeum decreases at 36h (Fig. 18C1, bracketed arrows, short arrow). The uniform epispheric expression develops into a specific mesodermal *Pdu-hes1* expression (Fig. 18C1, long arrows). *Pdu-hes1* is expressed (Fig. 18C2 and 3 arrows) around the chaetal sac anlagen (Fig. 18C2 and 3, arrowheads). The expression around the third chaetal sac anlagen is stronger than in the other two anterior pairs (Fig. 18C2 and 3, arrows). In contrast to the two anterior pairs, the

two presumptive chaetal sacs are completely surrounded by the expression domain (Fig. 18C2). Expression in the mesoderm instead of the ectoderm was determined due to the deeper localization of *Pdu-hes1*. It can not be detected at the surface of the embryos.

Twelve hours later, the expression in the episphere has almost disappeared and stomodaeal expression is limited to an area surrounding the mouth (Fig. 18D and E1). The expression around the chaetal sac anlagen has refined into a uniform pattern in and around the chaetal sacs but is limited to this lateral region (Fig. 18E1 and 2, bracketed arrows).

The *Pdu-hes1* expression around the chaetal sacs is sustained through the stage of 60h of development (Fig. 18F, small arrows). The most posterior expression in the hindgut (Fig. 18F, long arrow) can also be found at 72h (Fig. 18G1 and 2, long arrow). Together with the strong expression around the stomodaeum (Fig. 18G1 and F, small arrow), both regions of ectodermal gut development are labeled with *Pdu-hes1* expression.

At 4d, stomodaeal and hind gut expression domains have disappeared. Instead, both palp anlagen show expression (Fig. 18H, arrowheads). The posterior growth zone contains two domains of *Pdu-hes1* expression (Fig. H, arrows). Approximately 80 % (sample size N=50) of the animals in an in-situ hybridization experiment showed the described expression in the posterior growth zone. At 7d of development, the expression within the central nervous system has vanished, but *Pdu-hes1* is still expressed in two domains of the posterior growth zone (Fig. 18I1 and 2, arrows). This was observed in only 20 % of the juveniles. The remaining animals, 80 %, did not show any signs of expression. No *Pdu-hes1* expression could be detected in two, three and four week old juveniles.

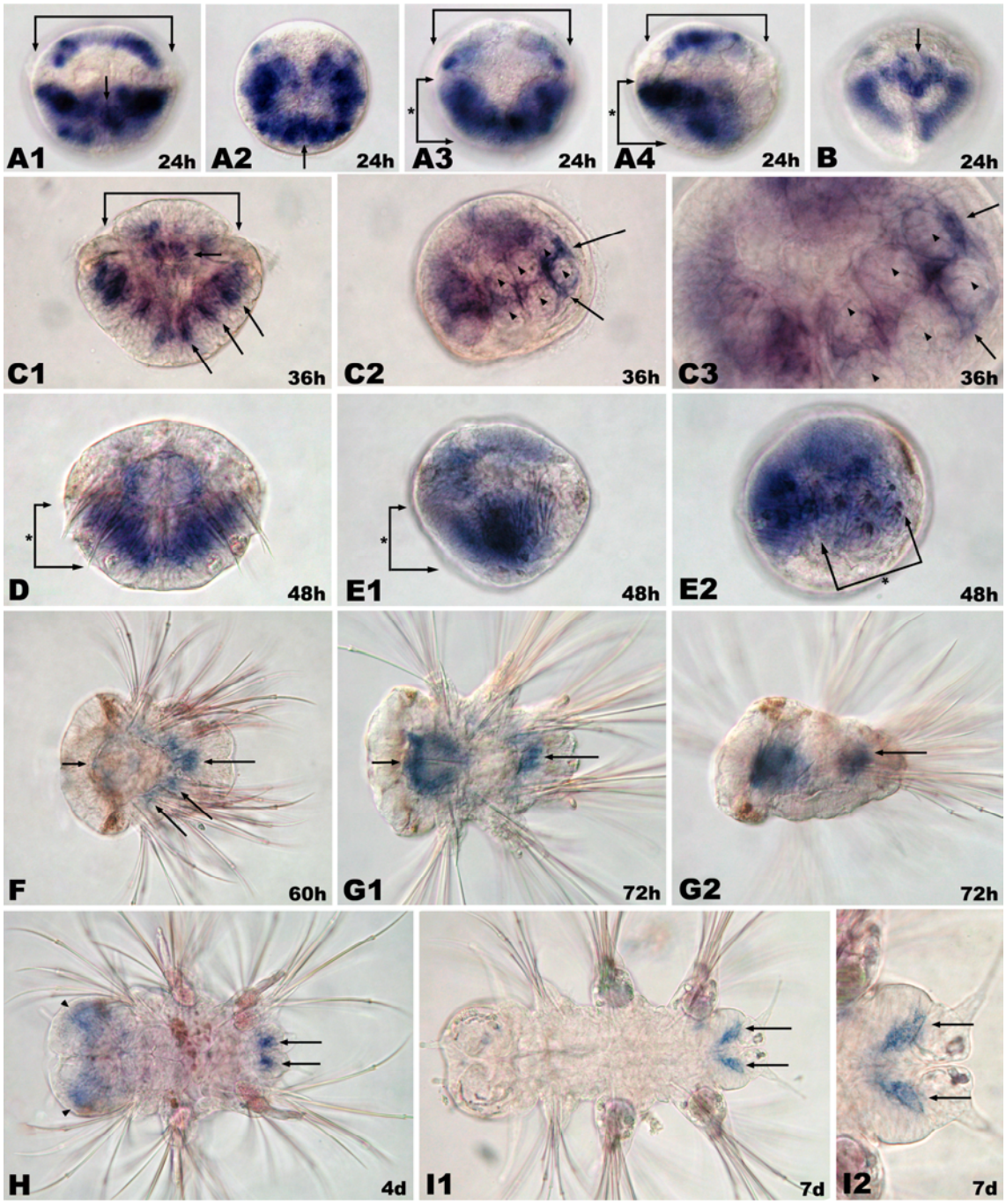


Fig. 18. ***Pdu-hes1*** **has various expression domains at different larval stages such as the central nervous system, the developing stomodaeum, the chaetal sacs and the posterior growth zone. A1, 2.** 24h; prominent expression of *Pdu-hes1* in the developing brain (bracketed arrows) and nearly the entire hyposphere including the stomodaeum (small arrow). **A3, 4.** 24h; epispheric (bracketed arrows) and hypospheric (asterisk bracketed arrows) expression of *Pdu-hes1*. **B.** 24h; stomodaeum expression (small arrow). **C1.** 36h; segmentally arranged expression of *Pdu-hes1* around the chaetal sac anlagen (arrows), transcript location in the mouth region (small arrow) and CNS (bracketed arrow). **C2.** 36h; defined expression (arrows) around the six chaetal sac anlagen (arrowheads) on each side of the larvae with close up (**C3**). **D.** 48h; extended *Pdu-hes1* staining in and around the chaetal sacs (**E1, 2**) (asterisk bracketed arrows). **F.** 60h; expression in the region of the developed chaetae decreases (arrows). **G1, 2.** 72h; *Pdu-hes1* transcript remains in the stomodaeum region (small arrow) and the ectodermal part of the hindgut (arrow). **H.** 4d; *Pdu-hes1* expression in the proposed pedipalp anlagen (arrowheads) and the posterior growth zone (arrows). **I1, 2.** 7d; isolated expression of *Pdu-hes1* in the posterior growth zone of juveniles (arrows) (**I2** close up). 24-48h: anterior is up, ventral down and dorsal up. 60h-3W: anterior is to the left, ventral down and dorsal up. A1,

C1, D, F, H and I1,2 are ventral views. A3, E1 and G2 are dorsal views. A2 is a posterior view, B a ventro-posterior view and A4 as well as E1 and G2 are lateral views. C2, 3 and E2 are ventrolateral views. A1-4, C1-3, E1, 2, G1, 2 and I1, 2 are the same animals. D is a merge of two foci of the same animal.

3.3.4 *Pdu-hes2*

The primers Csh-fw1, h-bw1 and h-bw2 (DAMEN ET AL. 2000) and mixed stage cDNA were used to perform a PCR. A putative 138 bp fragment of a *hes*-gene was isolated from the same PCR used to isolate *Pdu-hes1*. After RACE-PCR, the composite cDNA of 2225 bp resulted in a 1356 bp complete predicted ORF translating into 452 amino acids.

Following the phylogenetic analysis (Fig. 14), which places this gene at the base of the invertebrate *E(spl)* group and the *hairy* family, we called the gene *Pdu-hes2*. The position of the gene in this group could not be resolved with the three obtained trees. The sequence of *Pdu-hes2* contains all motifs typically for a bHLH-O gene like the bHLH-region, the Orange domain and the C-terminal tetrapeptide (Fig. 13). The mentioned conserved proline residue at the 5`end of the bHLH-domain (3.3.1) can also be found in the amino acid sequence of *Pdu-hes2*.

At 24h, prominent expression of *Pdu-hes2* can be detected lateral of the mouth in two vertical patches (Fig. 19A1, 3 and 4, short arrows). *Pdu-hes2* is also expressed in the central nervous system (Fig. 19A1, 3 and 4, arrowheads) and posterior to the mouth (Fig. 19A1 and 3, long arrows). The expression lateral of the stomodaeum is not only at the surface, but also in deeper layers of the tissue forming the mouth (Fig. 19A3 and 4, arrows). The dot-like expression of *Pdu-hes2* in the episphere is characterized by four patches, each four to five cells in wide (Fig. 19A5, arrows). They are more or less symmetrically arranged and could represent the development of special ganglia in the central nervous system as well as gland cells or sense organs. Two expression domains posterior to the mouth and probably lateral to the future anus (Fig. 19B1 and 2, arrows) could represent the described pigment area of Wilson´s fig. 83 in plate XX, (Wilson 1892).

Pdu-hes2 expression changes drastically in the next 12 hours. The signal is very weak in 36h stages. Faint expression in the ventral midline cells in the middle of the ventral plate can be detected (Fig. 19C1 and 2, arrow). This expression is sustained through 48h and 60h of development (Fig. 19D1 and 3, E1 long arrow; Fig. 19D2, bracketed arrows). At 48h, mouth expression is coming up in the inner circle of cells around the stomodaeum (Fig. 19D1-3, short arrow). The mouth expression becomes even stronger at 60h and is now

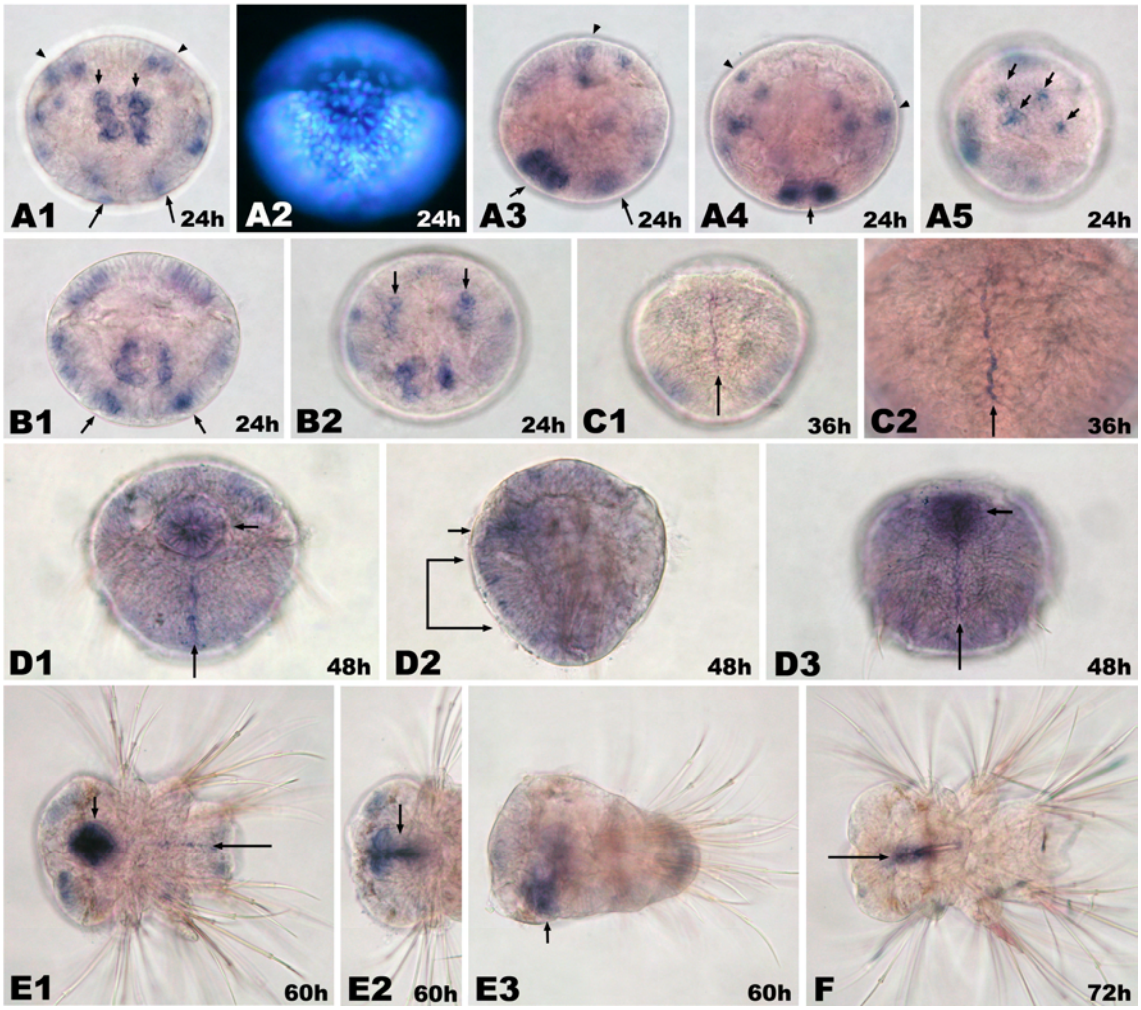


Fig. 19. *Pdu-hes2* seems to be a good marker for stomodaeum development. A1-4. 24h; prominent expression around the stomodaeum is visible in all views (small arrows), as well as expression posterior of the mouth (long arrows) and bilateral of the episphere (arrowheads). **A5.** 24h; lateral expression of *Pdu-hes2* in the episphere of the larvae (arrows). **B1, 2.** 24h; localization of the expression posterior to the stomodaeum (arrows). **C1, 2.** 36h; weak expression of *Pdu-hes2* in the so called "epithelial sheath cells" of the neural plate (arrow). **D1-3.** 48h; remaining mouth (small arrow) and sheath cell expression (long arrow/bracketed arrows in **D2**). **E1-3.** 60h; stomodaeum expression (small arrow) remains as well as the sheath expression (long arrow). **F.** 72h; defined stomodaeum region expression (small arrow). 24-48h: anterior is up, ventral down and dorsal up. 60h-3W: anterior is to the left, ventral down and dorsal up. A1, 2, C1, 2 and D3 are ventroposterior views. A3, D3 and E3 are lateral views. A4 is an apical view and A5 a lateral apical view. B2 is a posterior view. Ventral views are D1, E1,2 and F. A2 was exposed to Hoechst 33342. A1-5, B1, 2, C1, 2, D1-3 and E1-3 are the same animals.

located in the cells forming the anterior part of the mouth (Fig. 19E1 and 2, short arrow) as well as in the stomodaeum itself (Fig. 19E3, arrow). Twelve hours later in development, expression of *Pdu-hes2* is limited to the anterior part of the mouth (Fig. 19F, arrow). The expression in the epidermal sheath cells has disappeared. In 2, 3 and 4 week old juveniles, no expression of *Pdu-hes2* could be detected.

3.4 The modulator *fringe*

The glycosyltransferase *fringe*, which modulates *Notch* signaling (see introduction) (Maroto and Pourquie 2001; Panin et al. 1997), could only be isolated from *Capitella* sp. I.

A nested PCR approach with the listed primers (2.2.2.1.1) and *Capitella* sp. I cDNA library was used to isolate a 180 bp putative *fringe* fragment. The obtained 1621 bp cDNA resulted in a predicted ORF of 901 bp and translated into a 299 amino acid protein.

The translated protein, which we called *CapI-fringe*, was aligned to other *fringe* sequences from various taxa to demonstrate the high sequence conservation within the animal kingdom (Fig. 20). It can also be suggested, that the isolated *CapI-fringe* probably represents the so called mature *fringe* protein sequence. *Fringe* proteins have a general structure beginning with the pre-region or the signal peptide necessary for secretion, the pro-region containing a proteolytic site for cleaving and a mature region, which is the functionally active part of the protein (Wu et al. 1996). The pre and/or pro-region of *CapI-fringe* are missing and only the mature region could be isolated. Some *fringe* proteins only possess a pre-region (*manic-fringe* and *radical-fringe*) (Wu et al. 1996). So we do not know whether the isolated *CapI-fringe* actually has a pro- AND a pre-region.

CapI-fringe expression was only analyzed between stage 4 and late stage 6. Older as well as younger stages were not tested in this analysis.

At stage 4, the first signs of expression are detectable as a narrow line in the presumptive segmented tissue from anterior to posterior (Fig. 21A1-3, bracketed arrows). This lateral expression becomes stronger in early stage 5 (Fig. 21B1-3, bracketed arrows) and also expands dorsally during late stage 5 (Fig. 21D2, bracketed arrows). At stage 5, a second bilateral expression region comes up located in the most ventral parts of both belly plates spanning the first approximately three to four segments (Fig. 21E, long arrows). At late stage 5, this expression can be detected one to two segments towards the posterior of the larvae, but is still located at the ventral midline (Fig. 21D1, arrows). A few hours later in development, *CapI-fringe* is expressed in the first five to six segments in the most ventral part bilateral in both belly plates (Fig. 21E1, arrows). This expression has faded in stage 6 embryos, but the lateral expression of *CapI-fringe* has expanded dorsally and has become stronger (Fig. F1 and 2, bracketed arrows). The higher concentration of transcript in the posterior can already be seen, but is more apparent in late stage 6 larvae (Fig. 21G1 and 2, asterisk bracketed arrows) in comparison to the anterior lateral expression (Fig. 21G1 and

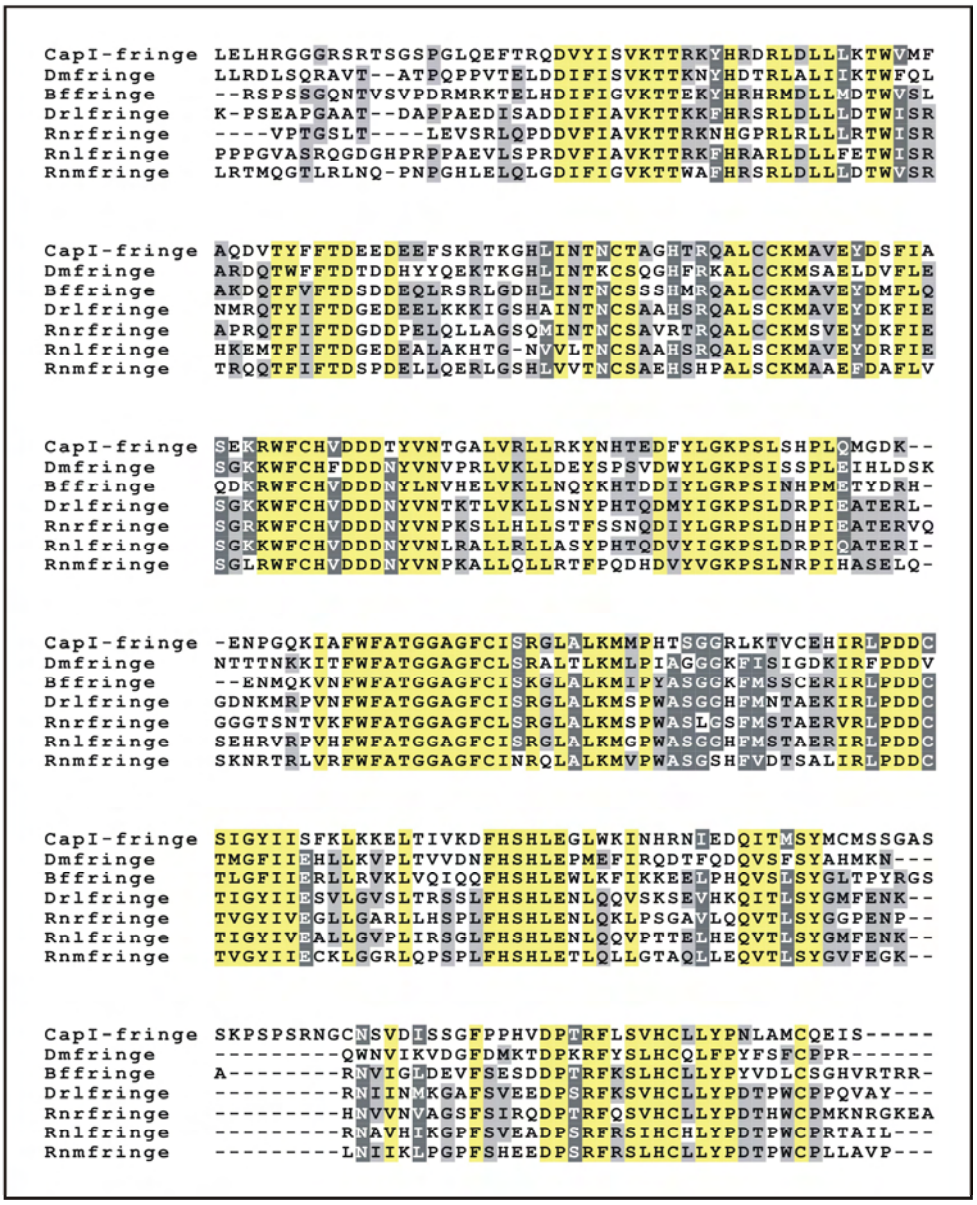


Fig. 20. Alignment of the *CapI-fringe* protein with different *fringe* sequeneces of different taxa. Dashes indicate gaps. Yellow highlighted amino acids label up to 100 % similarity, dark grey up to 80 % and grey up to 60 % identity. Dm-*Drosophila melanogaster*; Bf-*Branchiostoma florida*; Rn-*Rattus norvegicus*; *lfringe-lunatic fringe*; *rfringe-radical fringe; mfringe-manical fringe*.

2, bracketed arrows).

There is also prominent *CapI-fringe* expression in the central nervous system (Fig. 21A1, long arrow) and bilateral to the mouth (Fig. 21A1, C, short arrows) throughout all stages examined. The CNS expression is located in both cerebral ganglia (Fig. 21E3, arrows) and seems to be connected via the labeling of a cerebral commissure.

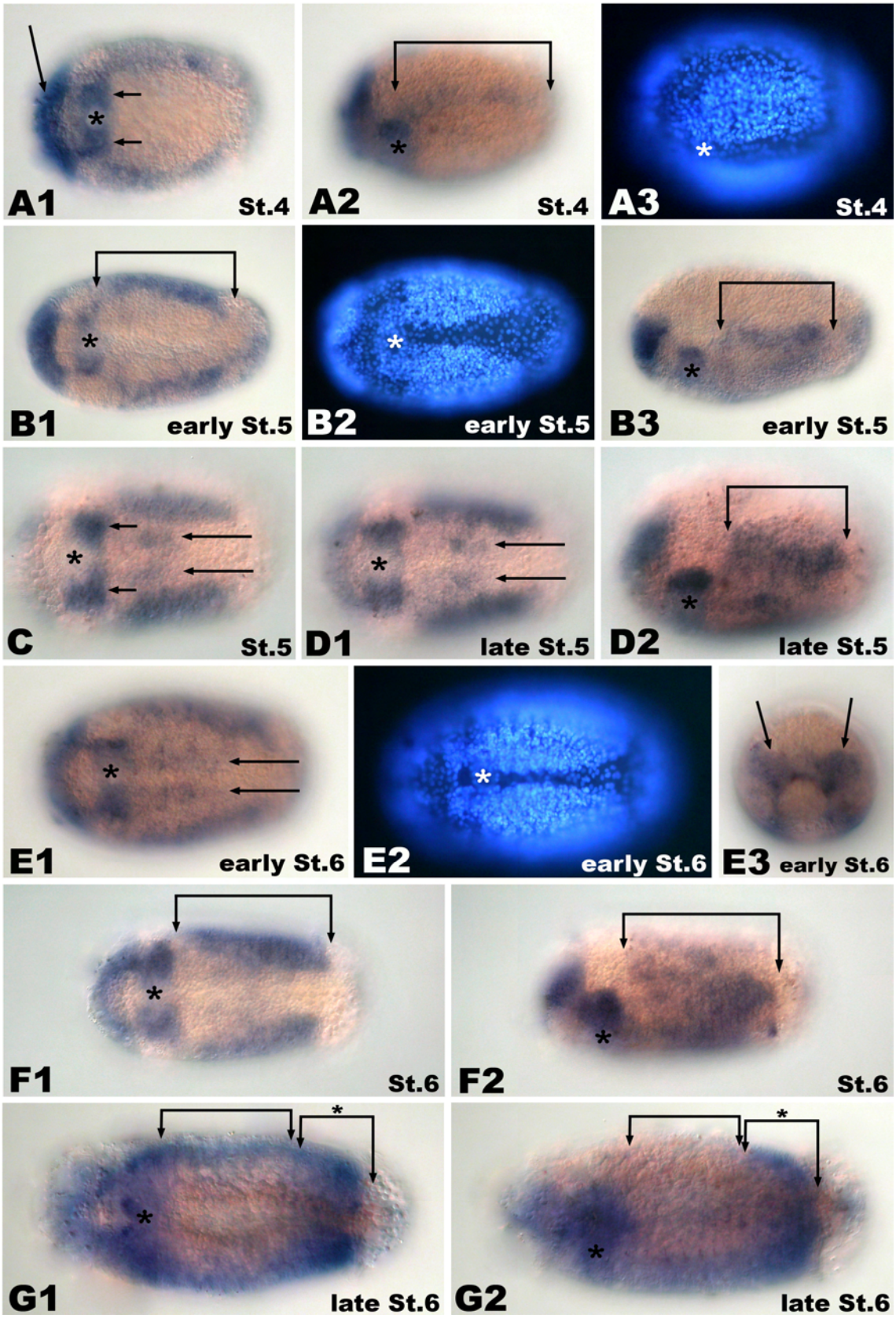

A1
St.4
A2
St.4
A3
St.4
B1
early St.5
B2
early St.5
B3
early St.5
C
St.5
D1
late St.5
D2
late St.5
E1
early St.6
E2
early St.6
E3
early St.6
F1
St.6
F2
St.6
G1
late St.6
G2
late St.6

Fig. 21. *CapI-fringe* is expressed in the segmented and unsegmented region of the presumptive segmented tissue, the brain and the developing foregut. A1-3. St. 4; first signs of *CapI-fringe* expression in the belly plates (bracketed arrows) as well es in the brain (long arrows) and the lateral region of the developing foregut (small arrows). **B1-3.** Early St. 5; belly plate expression expands (bracketed arrows). **C.** St. 5; appearance of a new expression domain at the ventral midline (long arrows); lateral foregut expression becomes more intense (small arrows). **D1.** Late St. 5; migration of ventral midline expression towards the middle of the larvae (long arrows). **D2.** Late St. 5; dorsal lateral expansion of fringe expression (bracketed arrwows). **E1, 2.** Early St. 6. broader expression of *CapI-fringe* in the ventral midline (long arrows). **E3.** Early St. 6; *CapI-fringe* transcript in the brain of the *Capitella* sp. I larvae (arrows). **F1, 2.** St. 6; intense expression of *CapI-fringe* in the formed and forming segments (bracketed arrows), as well as dorsal expansion of staining (F2). **G1, 2.** Late St. 6; decrease of expression in the anterior region of the larvae (bracketed arrows) and intense posterior *CapI-fringe* expression with expansion to the ventral midline (asterisk bracketed arrows). Anterior is to the left, dorsal up and ventral down for all pictures. A2, 3, B3 and D2 are ventro-lateral views. F2 and G2 are lateral views. A1, B1, 2, C, D1, E1, 2, F1 and G1 are ventral views. E3 is an apical view. A3, B2 and E2 were exposed to Hoechst 33342. A1-3, B1-2, D1, 2, E1, 2, F1, 2 and G1, 2 are the same animals. Asterisk marks the mouth. p-prototroch; t-telotroch.

3.6 The repressor *Suppressor of hairless Su(H)*

Su(H) or CSL (see introduction) was only isolated from *P. dumerilii*. A nested and a semi-nested RT-PCR were performed with the primers Su(H)-fw1, Su(H)-bw1 (Schoppmeier and Damen 2005) and Su(H)-Dr-fw2, Su(H)-Dr-bw3 (Sieger et al. 2003). Two putative *Su(H)* fragments of 240 bp and 575 bp were isolated. The 240 bp sequence was used to design RACE-primers. The 5' and 3' RACE-PCRs yielded a complete predicted ORF of 1455 bp and a deduced protein sequence of 485 amino acids. Even though the complete predicted ORF could be isolated, it was not possible to recover the entire cDNA of *Su(H)* in *P. dumerlii*. The poly-A tail, typical for the 3`end of the cDNA, was not found in the sequenced clones. The obtained cDNA sequence was 2701 bp long. The amino acid sequence of *Pdu-Su(H)* exhibits high similarity to many other *Su(H)* sequences from invertebrates as well as from vertebrates (Fig. 22). It does not contain certain conserved domains.

Expression of *Pdu-Su(H)* in 24h old embryos could not be detected (data not shown). First signs of expression are uniform and weak, visible in 36h larvae (Fig. 23A1 and 2).

Around 48h of development, *Pdu-Su(H)* expression becomes more precise and distinct with a V-shaped staining located near to the posterior edge of the ventral plate (Fig. 23B1-3, C, arrow). The expression is detected in deeper layers of the tissue (Fig. 23B2, arrow) and posterior to the mouth (Fig. 23C, arrow).

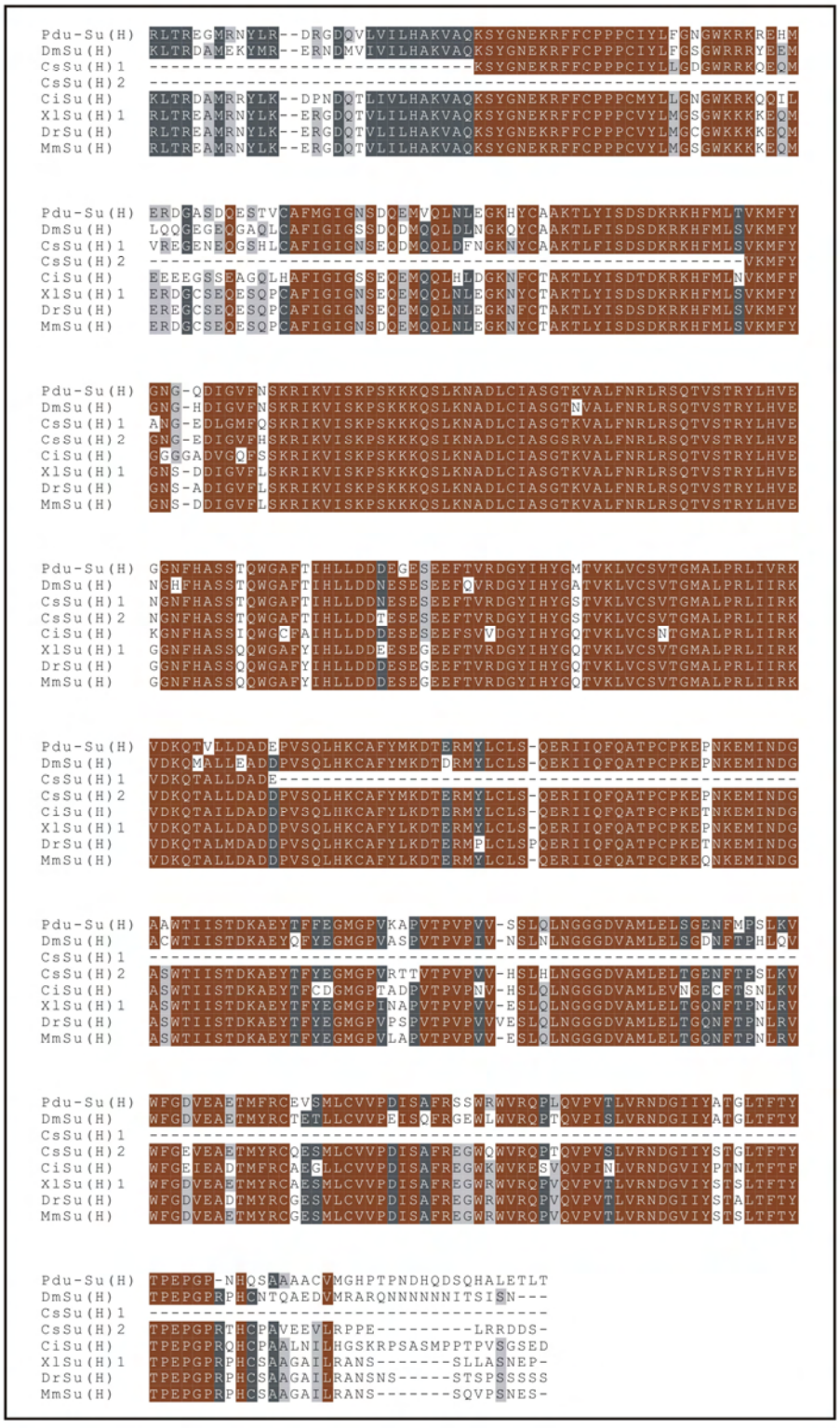


Fig. 22. Alignment of the *Pdu-Su(H)* amino acid sequence to other Su(H) sequences of various taxa. Dashes indicate gaps. Brown highlighted letters label identity of up to 95 %, dark grey of up to 80 % and grey of up to 60 %. Dm-*Drosophila melanogaster*; Cs-*Cupiennius salei*; Ci-*Ciona intestinalis*, Xl-*Xenopus laevis*; Dr-*Danio rerio*; Mm-*Mus musculus*.

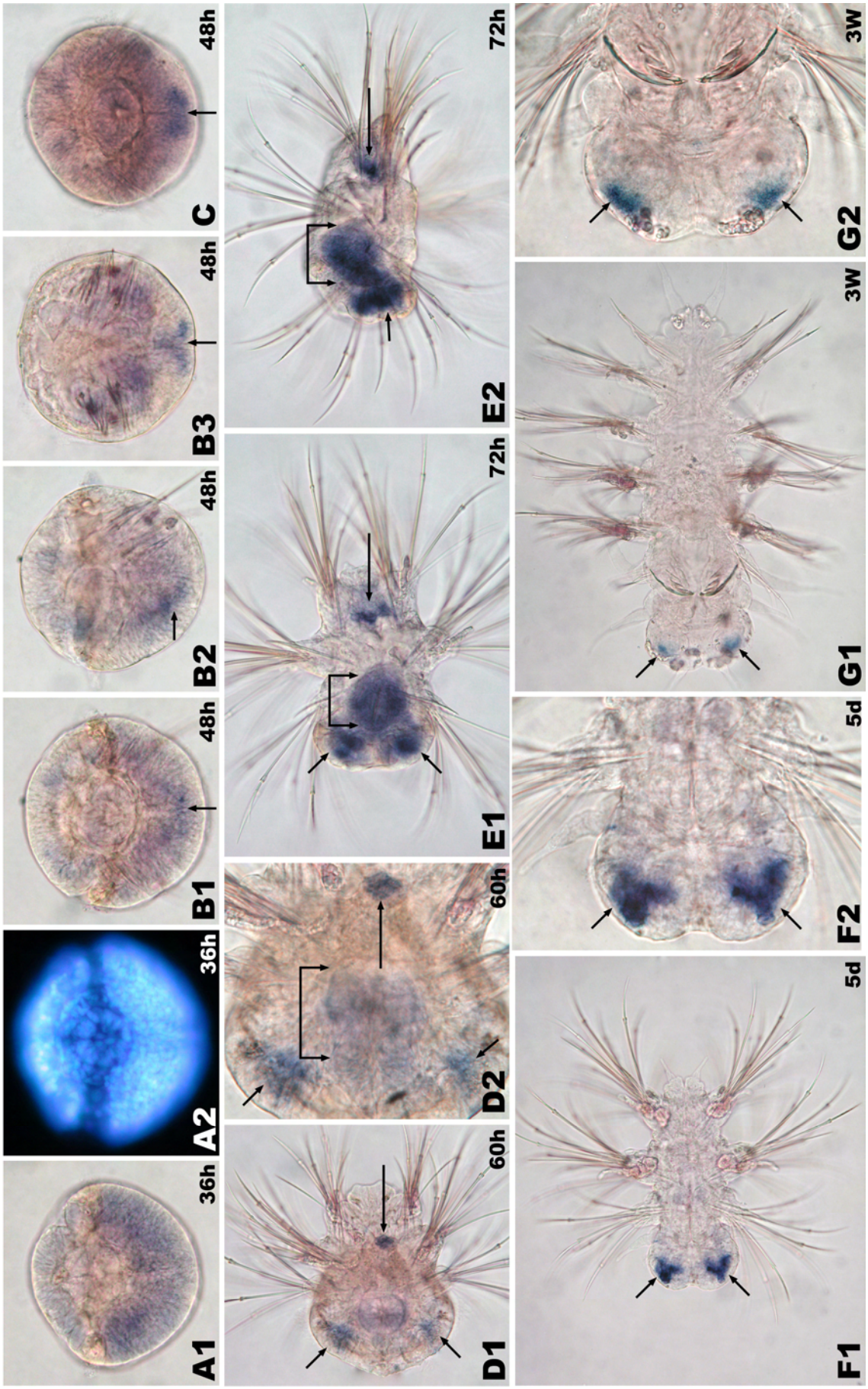

A1
36h
A2
36h
B1
48h
B2
48h
B3
48h
C
48h
D1
60h
D2
60h
E1
72h
E2
72h
F1
5d
F2
5d
G1
3W
G2
3W

Fig. 23. *Pdu-Su(H)* is expressed in the cerebral ganglia of *Platynereis dumerilii*. A1, 2. 36h; very vague and uniform expression in early stages. **B1-4.** 48h; V-shaped expression pattern right posterior to the stomodaeum (arrow). **D1, 2.** 60h; expression in the ganglia (small arrows) and probably in the hindgut (long arrow) as well as weak expression in the stomodaeum (bracketed arrows in **D2**). **E1, 2.** 72h; expression in the central nervous system (small arrows) and in the mouth (bracketed arrows) becomes stronger; hindgut expression seems to change (long arrow). **F1, 2.** 5d; ganglia expression remains very strong as the only expression domain (arrow) and decreases slighly over time, 3 W (**G1, 2** arrows). 24-48h: anterior is up, ventral down and dorsal up. 60h-3W: anterior is to the left, ventral down and dorsal up. B3 is a ventroposterior view. D2 and E2 are lateral views. A1, 2, B1, C, D1, 2, G1, 2 and I1, 2 are ventral views. A2 was exposed to Hoechst 33342. A1, 2, B1-3, D1, 2, E1, 2, F1, 2 and G1, 2 are the same animals.

Twelve hours later, *Pdu-Su(H)* expression has a completely different pattern than at 48h. There are precise regions of expression in the hindgut (Fig. 23D1 and2, long arrow), in the cerebral ganglia (Fig. 23D1 and2, short arrows) and the vague but broad stomodaeal expression (Fig. 23D2, bracketed arrows). At 72h, the *Pdu-Su(H)* pattern does not change, but the expression becomes stronger (Fig. 23E1 and2, long arrows, short arrows and bracketed arrows).

At 5d, the expression is limited to the cerebral ganglia (Fig. 23F1 and 2, short arrows) and this pattern is sustained over at least three weeks of development in juveniles (Fig. 23G1 and 2, short arrows). The level of expression is slightly lower in older stages, but all juveniles show *Pdu-Su(H)* expression.

3.7 The muscle marker *mef2*

After performing a semi-nested RT-PCR with the primers Psn-fw1, Psn-bw1 (Schoppmeier and Damen 2005) and Psn-bw0, a putative *mef2* fragment of 483 bp was isolated from *Platynereis*. By performing 5´RACE, a 2145 bp cDNA was obtained containing a 1386 bp complete predicted ORF, which translates into a 462 amino acid sequence. No 3`RACE was performed, because the 3`end of the ORF including some 3`UTR was recovered by degenerate PCR. The *Pdu-mef2* sequence contains a MADS domain and a *mef2* region (Fig. 24), both typical for all *mef2*-genes and necessary for specific DNA target binding (Leifer et al. 1993; Yu et al. 1992).

Pdu-mef2 expression in 24h embryos is very complex with various domains of ectodermal expression. *Pdu-mef2* is expressed around the posterior stomodaeum in a sickle shaped fashion (Fig. 25A1, arrow). The expression of *Pdu-mef2* within the central nervous system of the embryo is very broad and stretches through almost the entire epispheric ectoderm (Fig. 25A1, bracketed arrows). There is a line of expression, which is one to two cells wide along the dorsoventral axis (Fig. 25A2, long arrow). This line of expression is located right

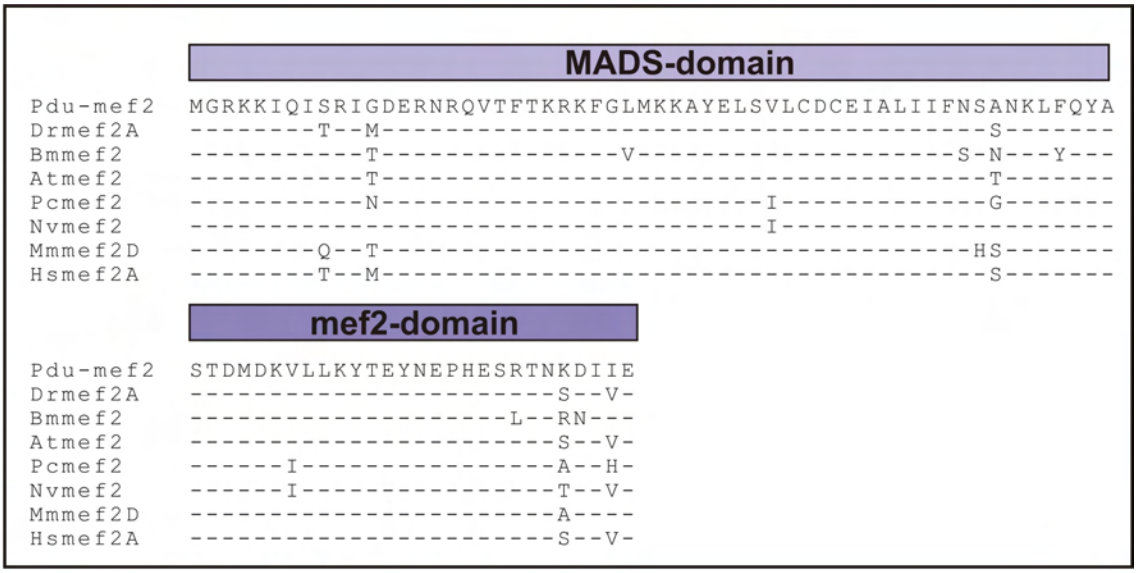


Fig. 24. Alignment of the MADS and *mef2*-domain of different *mef2*-protein sequences including *Pdu-mef2*. Dashes indicate identical amino acids. Dr-*Danio rerio*; Bm-*Bombyx morii*; At-*Achaearanea tepidariorum*; Pc-*Podocoryne carnea*; Nv-*Nematostella vectensis*; Mm-*Mus msculus*; Hs-*Homo sapiens*.

under the prototroch of the embryo and might mark the development of the lateral region of the so called peristomial segment. The dorsal view shows the bilateral lines right under the prototroch (Fig. 25A3, arrows). Another characteristic of the *mef2*-expression in *P. dumerilii* are two dots located bilaterally at the posterior-dorsal side of the embryo (Fig. 25A4, arrows). They are probably right next to the future anus.

Starting at 36h of development, the expression pattern changes gradually and drastically. The only expression which could be detected is located in the mesoderm (Fig. 25B1, arrows) in the presumptive muscles around the chaetal sac anlagen (Fig. 25B2, C bracketed arrows). The future chaetae are not completely surrounded by the *Pdu-mef2* expression. There are gaps visible, especially in the more ventral row of chaetae at the ventroposterior side (Fig. 25B2, bracketed arrows, Fig. 25B3). The exact differences in expression between the six chaetae on each side of the embryo are very individual and depend on the larva itself (compare Fig. 25B2 and3 and Fig. 25C).

The expression in the mesoderm expands rapidly over time (Fig. 25D1, arrows). At 48h, *Pdu-mef2* is expressed in the muscles around the already formed chaetae on each side of the embryo (Fig. 25D2 and D3, bracketed arrows). The first two pairs of chaetae are completely encircled with *Pdu-mef2* expression. The last pair on each side of the embryo lacks expression at the most dorsal side of the embryo (Fig. 25D4, arrow).

The mesodermal *Pdu-mef2* expression around the chaetae is sustained through 60h of development, but becomes weaker (Fig. E1 and 2, bracketed arrows). At 72h, the

remaining expression of *Pdu-mef2* is detected anterior to the second and third pair of chaetae (Fig. 25F, arrows) on each side of the embryo.

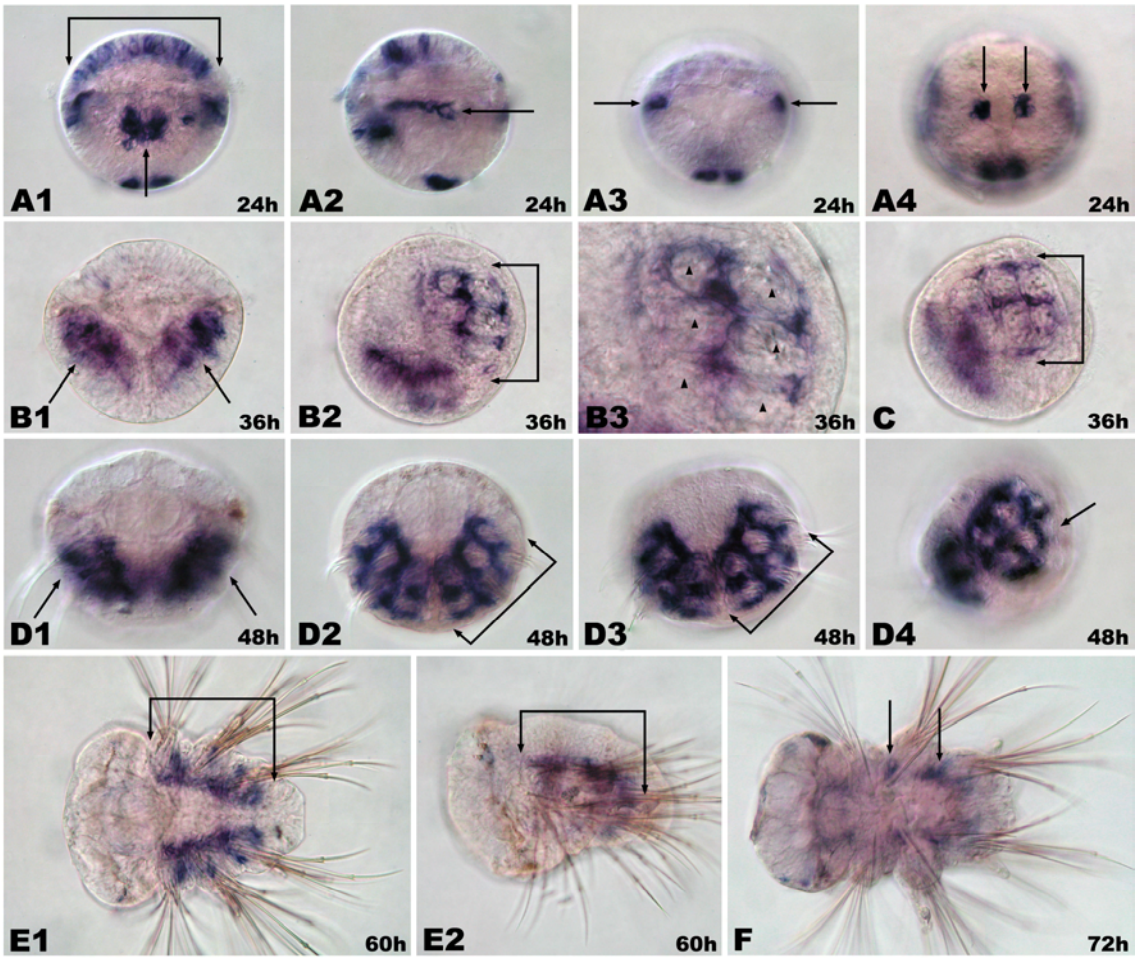


Fig. 25. *Pdu-mef2* is expressed around the chaetae in the presumptive muscle tissue. A1. 24h; expression around the stomodaeum (arrow) and the developing CNS (bracketed arrows). **A2.** *Pdu-mef2* is also expressed in the lateral region of the so called peristomial segment (arrow), bilaterally visible in the dorsal view (**A3**, arrows). **A4.** 24h; two dots of expression at the posterior of the embryo to the left and right of the future anus (arrows). **B1.** 36h; *Pdu-mef2* expression at both sides of the embryo in the developing muscles (arrows). **B2.** 36h; transcript localized in the muscles (bracketed arrows) around the more posterior lying chaetae (arrowsheads, **B3**, close up). **C.** 36h; individual differences in lateral expression (bracketed arrows). **D1.** 48h; stronger expression in the muscles (arrows). **D2, 3.** 48h; *Pdu-mef2* around all six chaetae on each side (bracketed arrows). **D4.** 48h; nearly no expression at the lateral posterior of the third chaetae pair (arrow). **E1, 2.** 60h; sustained expression around the already formed chaetae (bracketed arrows). **F.** 72h; weaker expression in the muscles in later stages (arrows). 24-48h: anterior is up, ventral down and dorsal up. 60h-3w: anterior is to the left, ventral down and dorsal up. A1, B1, D1, E1 and F are ventral views. A2, C and E2 are lateral views; B2, B3, D3 and D4 are ventrolateral views. A3 is a dorsal view, A4 a posterior view and D2 a ventroposterior view. A1-4, B1-3, D1-4 and E1, 2 are the same animals. D1 is a merge of two foci of the same animal.

3.8 Juvenile expression of *Notch, Delta* and *hes*-genes in *Capitella* sp. I

The following results were generated by Elaine C. Seaver with the isolated genes described in this thesis except for *CapI-hes2* and *CapI-hes3* found in the trace files of the *Capitella* sp. I genome. They are briefly presented and described for the purpose of the discussion of the results.

After larval segmentation, the addition of segments by the posterior growth zone in juveniles follows. The posterior growth zone in *Capitella* sp I can be devided into a subterminal region and the anterior to that located nascent segment. *CapI-Delta* and *CapI-Notch* are mainly expressed in the mesoderm and to a lesser extend in the ectoderm of the nascent segment and in the anteriorly located newly formed segment (Fig. 26A and D, arrows). In contrast, *CapI-hes1* is expressed in the mesoderm of the subterminal zone of the posterior growth zone (Fig. 26G, arrow). *CapI-hes2* and *CapI-hes3* are located in the mesoderm and ectoderm of the entire growth zone including the nascent segment and the subterminal zone as well as the last formed segment (Fig. 26J and N, arrows). The exact localization of every transcript in one or two germ layers can be seen in the lateral view of every gene (Fig. 26C, F, I, M, P) or the red/blue pseudocolor/Hoechst pictures (Fig. 26B, H, K, O). *CapI-Notch, CapI-Delta* and *CapI-hes2* also show expression in the ganglia of the ventral nervous system (Fig. 26C, E, F, J, diagonal arrows).

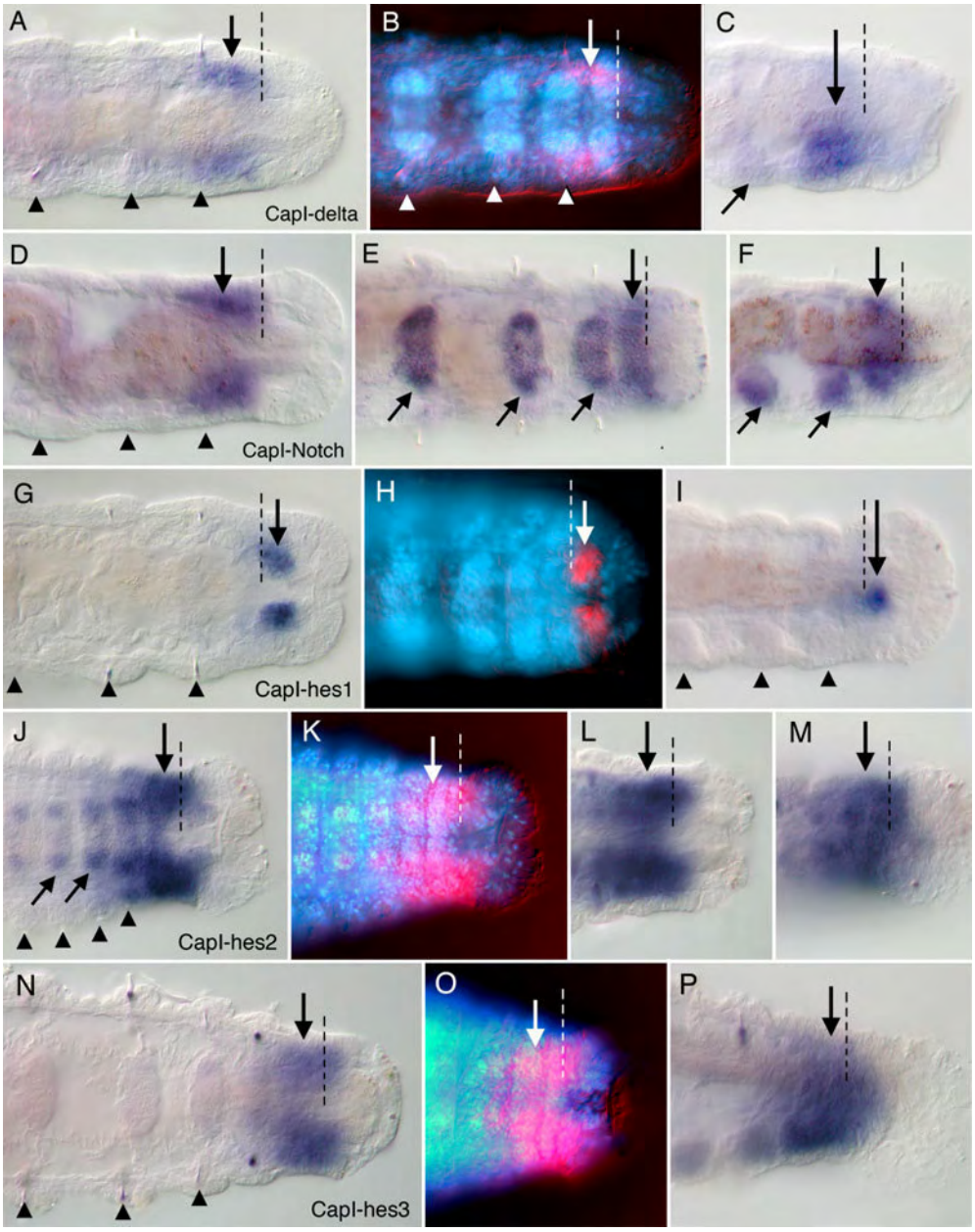


Fig. 26. Expression of *CapI-Delta*, *CapI-Notch* and *CapI-hes* genes in juveniles of *Capitella* sp. I. (E.C. Seaver, unpublished results). **A-C.** *CapI-Delta* is expressed in the mesoderm of the nascent segment and the adjacent anterior segment (arrow). **D-F.** *CapI-Notch* expression is localized in the mesoderm and ectoderm of the nascent segment and the adjacent anterior segment (vertical arrow). **G-I.** *CapI-hes1* expression is restricted to the mesoderm of the subterminal region in the posterior growth zone (arrow). **J-M.** *CapI-hes2* is expressed in the ventral ganglia (diagonal arrows), in the subterminal region and the nascent segment of the growth zone. The expression located in the ectoderm and mesoderm becomes gradually weaker from anterior and posterior to the terminal growth zone. **N-P.** *CapI-hes3* expression is located in the ectoderm and mesoderm of the posterior growth zone including the subterminal region and the nascent segment and a few segments anterior to the nascent segment (vertical arrow). Anterior is to the left for all panels. A, B, D, E, G, J, K, L, N and O are ventral views. H is a dorsal view. C, F, I, M and P are lateral views with ventral down. B, H, K and O are Hoechst images overlain with the DIC image of the expression domains in red pseudocolor (Photoshop) to show localization of expression domains within the growth zone. Vertical dashed line marks the posterior boundary of the nascent segment. Diagonal arrows mark the ventral ganglia. Arrowheads mark the chaetae.

4. Discussion

Displaying a membrane bound receptor and ligand, the *Delta-Notch* signaling pathway requires cell-cell contact. The expression of both genes can be expected in the same regions and also neighboring cells even though one of the components can be expressed broader than the other factor. In contrast, we can assume that one component displays the inductive and limited expressed factor comparable to i.e. somitogenesis in vertebrates. During this process, *Notch* is usually expressed almost uniform in the PSM while the *Delta* expression exhibits a striped like pattern in this region (Bettenhausen et al. 1995; Pourquie 2001; Williams et al. 1995).

Since no *Notch* independent function of the regulator *fringe* has been discovered so far, it can be assumed, that the expression of both genes overlaps to a certain extend such as in the dorsal/ventral wing margin formation in *Drosophila*. Both cell types express *Notch*, while only the dorsal cells express *fringe* to inhibit the binding of the ligand *Serrate* to its receptor *Notch* (Irvine and Wieschaus 1994; Johnston et al. 1997; Panin et al. 1997).

Hes/hairy genes are downstream targets of *Notch*. They are activated by the binding of the NICD to a nuclear repressor, which then becomes an activator and transcription of the *Enhancer of split* genes occurs (see Introduction) (Jarriault et al. 1995; Takke and Campos-Ortega 1999). Therefore, one would expect to see co-expression of *Notch* and *hes* genes even though certain *hes* genes can also act independent of *Notch* i.e. to maintain stem cell character of nervous cells (Kageyama et al. 2007).

Assuming the conservation of the canonical *Notch* pathway in polychaetes, we can expect an overlapping expression of *Pdu-Notch* and *Pdu-Su(H)*, but latter gene can also be expressed in *Notch* negative cells to repress downstream targets of *Notch* not being activated in those cells (Fortini and Artavanis-Tsakonas 1994).

An overlap of expression of *Pdu-Notch* and *Pdu-mef2* is not necessarily the case, because *mef2* is not a direct downstream target of *Notch* in other organsims, but is activated by other factors influenced by the *Notch*-signaling (Shen et al. 2006).

4.1 Spatial and temporal relationship of the *Capitella* sp. I gene expression patterns of *CapI-Notch, CapI-Delta, CapI-hairy and CapI-fringe*

For *Capitella* sp. I, embryos younger than St. 4 were not examined. *CapI-Notch* and *CapI-Delta* have very similar and overlapping expression patterns in larval as well as in juveniles stages. In larvae, both genes are initially expressed in the ectoderm of the presumptive segmented tissue. *CapI-Notch* has a much broader expression while *CapI-Delta* is expressed in a discrete cells or small clusters of cells in the belly plates of the larvae. *CapI-Notch* also shows limited expression in the mesoderm beneath the ectodermal transcripts in the bilateral unsegmented and segmented tissue. From St. 6 on, *CapI-Notch* and *CapI-Delta* are expressed in a two row pattern of segmentally iterated patches of cells at the position of all future chaetal sacs. At this stage of development, *CapI-Notch* is also expressed in a broader area compared with its ligand *CapI-Delta*, and the two rows of expression are also detectable. Additionally, both genes are expressed in the CNS and in two patches lateral to the mouth. In later stages, both genes are expressed in the mesoderm of the posterior growth zone and to a lesser extent in the ectoderm.

The juvenile expression of both genes can be detected in the nascent segment, the adjacent segment anterior of the posterior growth zone (E.C. Seaver, unpublished results). The juvenile growth zone in *Capitella* sp. I was defined as a subterminal zone plus the last formed or nascent segment. *CapI-Notch* and *CapI-Delta* are mainly expressed in the mesoderm and to a lesser extent in the ectoderm.

The similar expression patterns of *CapI-Notch* and *CapI-Delta* across developmental stages and distinct tissues in *Capitella* sp. I are consistent with these genes acting together within a ligand-receptor signaling network (Fehon et al. 1990; Heitzler and Simpson 1993; Rebay et al. 1991). The overlapping expression patterns of both genes in the presumptive chaetal sac anlagen before the formation of chaetae also suggest an involvement of the signaling pathway in the chaetogenesis of *Capitella* sp. I.

CapI-fringe expression coincides with the broad transcript localization of *CapI-Notch* throughout the stages it is expressed. All three characteristic expression domains, the CNS, the belly plates and the foregut, can be detected in both genes. The refinement of *CapI-Notch* expression and especially of *CapI-Delta* expression later on into two rows of dots at the position of the prospective chaetal sacs cannot be observed for the *CapI-fringe*

transcript. This can be explained with the proposed function of *fringe* in other organisms. As described previously (see Introduction), *fringe* seems to be a regulator of the *Notch* signaling pathway (Moloney et al. 2000). Depending on the ligand, it has a positive or a negative effect on the signaling process (Panin et al. 1997). Especially the border formation in diverse tissues is dependent on the influence of *fringe* and the *Notch* pathway (Irvine and Rauskolb 2001; Rauskolb et al. 1999). The overlapping expression patterns of *CapI-Notch* and *CapI-fringe* are typical for the functionality of *fringe*. Thus, it can inhibit other *Notch* ligands like *Serrate* or potentiate the binding of *Delta* to *Notch* (Haltiwanger 2002). Both ligand and receptor are chemically modified by *fringe*. This would also explain the overlapping expression of *CapI-Delta* and *CapI-fringe*. The *fringe* gene modulating the *Notch* signaling was only isolated from *Capitella* sp. I, but not from *P. dumerilii*.

The persistent broad expression of *CapI-fringe* without the described later more restricted pattern can be caused for example by a negative regulation of a second *CapI-Notch* ligand by *CapI-fringe* (Panin et al. 2002), like a *Serrate* homologue. *CapI-fringe* expression in St. 8 to 9 is missing in this analysis. It would be interesting to observe this expression pattern in older larvae of *Capitella* sp. I to see a possible transcript location in the terminal growth zone besides the *CapI-Notch* and *CapI-Delta* expression. This would suggest an influence of *CapI-fringe* in the later *Notch* signaling pathway too.

In early stage larvae, *CapI-hes1* has a very restricted expression pattern in contrast to *CapI-Notch*, *CapI-Delta* and *CapI-fringe*. The transcript is always associated with the unsegmented mesodermal part of the belly plates and in older larval stages is localized to the terminal growth zone with *CapI-Delta* and *CapI-Notch*. In juveniles, *CapI-hes1* is expressed in the subterminal region of the mesodermal posterior growth zone and to a small extent in the nascent segment (Fig. 26G-I). Thus, *CapI-hes1* expression coincides with the *Delta* and *Notch* expression of *Capitella* sp. I to a really small extent in the nascent segment and the subterminal region even though the latter two genes are expressed mainly anterior to *hes1*. The overlapping expression domains suggest that *CapI-Notch* and *CapI-hes1* could be expressed in the same cells. This might imply a possible activation of *hes1* by *Notch* in *Capitell*a sp. I. during late larval and juvenile development.

The expression pattern of *CapI-hes1* does not support any involvement in the formation of chaetae in comparison to *CapI-Notch* and *CapI-Delta*. *Hes/Enhancer of split* genes are often downstream targets of the *Notch* signaling pathway (Bessho et al. 2001; Iso et al.

2003; Kageyama et al. 2007; Takke and Campos-Ortega 1999). Our data for the juvenile expression of *CapI-Notch* and *CapI-hes1* are consistent with this. In contrast to the later stages of *Capitella* sp. I development, the earlier embryos and larvae do not show a significant overlap or co-expression of *CapI-Notch* and *CapI-hes1*. *CapI-Notch* is only expressed weakly in the mesoderm of the belly plates which might be sufficient to activate and regulate *CapI-hes1*. Another possiblity is, that *CapI-hes1* is a *Notch*-independent transcription factor in this stage of development and is not being regulated by this signaling pathway. *Notch*-independent activation of *hes* genes has been well documented as was already shown for developmental processes of various organisms (Bae et al. 2005; Hirata et al. 2001). Moreover, it can not be excluded, that a second or even more *Notch* genes exist in *Capitella* sp. I. We can assume a coexpression of the second *CapI-Notch* gene with *CapI-hes1* and thus it might also be involved in the regulation of *CapI-hes1*. An overlap of expression with *CapI-hes2* and *CapI-hes3* is also possible.

CapI-hes2 as well as *CapI-hes3* show prominent overlap of expression with *CapI-Notch* and *CapI-Delta* during St. 4/5 until St. 9 as well as during development of juveniles consistent with the conservation of the canoncial *Notch* pathway. Both *hes* genes could represent downstream targets for *CapI-Notch*, especially *CapI-hes2* with its prominent expression in the future chaetal sacs. Other overlapping expression domains such as the foregut, the developing brain and later on the posterior growth zone also account for this downstream signaling.

4.2 Spatial and temporal relationship of the *Platynereis dumerilii* gene expression patterns of *Pdu-Notch, Pdu-Delta, Pdu-hes1, Pdu-hes2 and Pdu-Su(H)*

Each of the early transcript localizations of all five genes is unique. Expression occurs in the CNS, the stomodaeum, larval eyes or the ventral plate. Analyses of gene expression patterns before 24h of development were not performed since an appropriate protocol for a reliable analysis expression for stages younger than 24h of development has not yet been established.

The early expression of *Pdu-Notch* overlaps with the 24h of *Pdu-Delta* expression. The uniform and very broad expression of *Pdu-Notch* at 24h makes that fact obvious. The overlapping expression of *Pdu-Notch* and *Pdu-Delta* in the presumptive chaetal sac anlagen and later on in the already formed chaetal sacs is very apparent at 36h and 48h of development. First signs of chaetae development can be seen between 30 and 36h of development. *Pdu-Notch* shows broader and a more extensive expression pattern in all three pairs of chaetal sac anlagen which later on refines into smaller patches. In contrast, *Pdu-Delta* shows expression in four of six presumptive chaetal sacs on each side of the embryo and later on in five of six chaetae. In both expression patterns in the described stages, the ventral row of chaetae shows stronger expression than the dorsal one, whereas the most-dorsal posterior chaetal sac shows almost no expression of *Pdu-Notch* nor of *Pdu-Delta*. One explanation for that can be the cephalization process later on during the further development of *P. dumerilii.* The first parapodia pair is incorparated into the head including the first notopodium. The second and third notopodium will form glands necessary for the production of polychaete silk of the living tubes (Fischer and Dorresteijn 2004; Hauenschild 1969).

Later from 60h of development on, both genes are expressed in the anterior portion of the mouth, whereas *Pdu-Notch* also shows expression lateral to the mouth opening. *Pdu-Notch* and *Pdu-Delta* also are expressed in the mesoderm of the posterior growth zone, which might imply an involvement in the later segmentation process. The overlapping localization of both transcripts in the same tissue and the same developmental stages is consistent with a possible receptor-ligand interaction of *Pdu-Delta* and *Pdu-Notch* in that particular tissue. The expression of both genes before and during the development of the

three chaetae pairs on each side of the larvae also suggest an involvement of *Notch* and *Delta* in the chaetogenesis of *P. dumerilii*.

Pdu-Delta expression is restricted to the chaetal sacs, while *Pdu-Notch* is also expressed in the CNS and around the stomodaeum at that time of development. *Pdu-Delta* shows no expression in the CNS and is very restricted in its expression in general. The absence of *Pdu-Delta* expression in certain domains of *Pdu-Notch* expression makes it very apparent, that a second *Pdu-Delta* gene exists. This gene is also supposed to work as a ligand for *Notch*. The second *Pdu-Delta* gene could be involved in the neurogenesis of *P. dumerilii* since *Pdu-Delta* itself is not expressed in the CNS. The existence of more than one *Delta* gene in one organisms was shown in several animals such as in the spider (Stollewerk et al. 2001), *Caenorhabditis elegans* (Gao and Kimble 1995) or most vertebrates (Bettenhausen et al. 1995; Deblandre et al. 2001; Dunwoodie et al. 1997; Jen et al. 1997). Therefore, it is not too suprising that the receptor *Notch* is expressed more broadly than one of its ligands *Delta.*

Pdu-hes1 is expressed very broadly early on and overlaps with *Pdu-Notch* expression. At 36h, there is more limited expression pattern in the mesoderm around the chaetal sacs. Twelve hours later, the *Pdu-hes1* has such a broad expression domain in and around the chaetal sacs that it overlaps with the transcripts of *Pdu-Notch* and *Pdu-Delta*. In later stages, *Pdu-hes1* is also expressed in the mesoderm of the terminal growth zone like *Pdu-Notch* and *Pdu-Delta*. In contrast to these two genes, *Pdu-hes1* expression can also be detected in later juvenile stages (day 7 of development). Only 20 % of the seven day old juveniles in one in-situ hybridization showed expression in the terminal growth zone. This might imply a dynamic expression of *Pdu-hes1* in the terminal growth zone during the later segmentation process. The stomodaeum expression of *Pdu-hes1* is consistent with the *Pdu-Notch* mouth expression and disappears around the same time of development for both genes.

Pdu-hes2 expression also colocalizes with the *Pdu-Notch* transcript in the early development of *P. dumerilii*. Later on, *Pdu-hes2* expression is only overlapping with *Pdu-Notch* in the expression domain of the stomodaeum. The expression of *Pdu-hes2* in the mouth persists from stages 24h through 72h old larvae. The *Pdu-hes2* expression in the so called epidermal sheath cells is probably a *Notch*-independent developmental process, which has been described in other systems (Kageyama et al. 2007). The existence of a second *Notch* gene in *P. dumerilii* cannot be excluded, which would then overlap with

Pdu-hes2 in its expression. The function of the *Pdu-hes2* transcript localization is hard to predict. Since this expression is ectodermal, *Pdu-hes2* could be involved in the formation of certain parts of the ventral nervous system or other neurogenic processes.

The repressor gene *Su(H)* could only be isolated from *P. dumerilii* and not from *Capitella* sp. I. *Pdu-Su(H)* expression could not be detected earlier than 36h. At this stage of development, Pdu-Su(H) is expressed uniform throughout the embryo. The expression pattern at 48h is also very weak and almost uniform throughout the embryo except for a very precise v-shaped expression posterior to the mouth.. *Pdu-Su(H),* like *Pdu-hes2,* shows no expression associated with the formation of chaetae in *P. dumerilii*. The transcript seems to be located very deep in the ectoderm or even in the mesoderm. This would have to be confirmed by studying sections of the stained embryo. At this stage of development, *Pdu-Su(H)* expression does coincide with *Pdu-Notch* expression or even with *Pdu-Delta* if its expression is only very weak.

Later expression of *Pdu-Su(H)* at 60h is possibly restricted to the ectoderm. *Pdu-Su(H)* and *Pdu-Notch* are expressed in the CNS and the stomodaeum. *Pdu-Su(H)* is also expressed in the posterior end of the hindgut. The posterior expression in 72h old larvae seems to overlap with the *Pdu-hes1* expression at the same stage. The mesodermal expression of *Pdu-Delta* and *Pdu-Notch* in the posterior growth zone at that stage of development is located posterior to the expression of *Pdu-Su(H)* and *Pdu-hes1* in the third segment. An overlap of expression for *Delta/Notch* and *Su(H)/hes1* can not be identified convincingly. A double in-situ hybridization would be necessary to resolve this issue. *Pdu-Su(H)* was the only gene in *P. dumerilii* detected in stages older than seven days. The expression of *Pdu-Su(H)* is located exclusively in the CNS from 5 days through 3 weeks of development.

As a conserved component of the *Notch* pathway, one would expect an overlap of expression with *Pdu-Notch* throughout the stages. No overlap of *Pdu-Su(H)* and *Pdu-Notch* expression in different developmental stages of *P. dumerilii* could be explained with the repressor function of *Su(H)* genes while the *Notch* signaling is not active in those cells. Secondly, it is possible that a *Notch* independent mechanisms of *Pdu-Su(H)* is acting in those regions like reported in *Drosophila* (Koelzer and Klein 2003) where it prevents the further development of cells to undergo a determined fate during the formation of mechanosensory bristles. Third, it might be that another *Notch* receptor in *P. dumerilli* exists and activates the repressor in the regions expressing *Pdu-Su(H)*. Since the *Suppressor of hairless* gene is a very conserved component of the *Notch* signaling pathway

(Lecourtois and Schweisguth 1997), it is also possible that a second *Pdu-Su(H)* gene exists, which regulates the other processes of development and shows other overlapping expression with *Pdu-Notch*. A second copy of the *Su(H)* gene has been found in several organisms like in the zebrafish (Wettstein et al. 1997) or in *Cupiennius salei* (Schoppmeier and Damen 2005). Strinkingly, the early and also later expression pattern of *Pdu-Su(H)* shows similarities to the expression in the spider. Early stages also display uniform expression of both *Su(H)* transcripts in this basal arthropod. Later on besides the ubiquitous expression, there are stronger expression domains in the head lobe, heart precursors, forming appandages and neuro-ectodermal tissue comparable to this v-shaped stronger expression of *Pdu-Su(H)* in the ventral plate at 48h of development. This also resembles the situation in *Drosophila* or vertebrates where *Su(H)* genes are upregulated in some tissues later on (Oka et al. 1995; Sieger et al. 2003).

4.3 Comparative analysis of the expression patterns of *Notch* pathway components in *Capitella* sp. I and *Platynereis dumerilii* genes

4.3.1 The ligand *Delta*

We recovered the complete predicted ORFs of two *Delta* genes, *CapI-Delta* and *Pdu-Delta* containing all characteristic domains of *Delta* genes (Fleming 1998). Both sequences contain all characteristics typical for a *Delta* gene like the DSL region, the EGF repeats and the transmembrane and intracellular region. The DSL region is a unique modified EGF repeat (Henderson et al. 1994; Tax et al. 1994) conserved in the *Notch* ligands *Delta, Serrate* and *C.elegans Delta lag-2*. It displays an indispensable sequence component for binding to its receptor *Notch. C. elegans* mutants missing the DSL region of *lag-2* show a loss-of-function phenotype (Henderson et al. 1997). Yuan and collegues showed, that this conserved domain is also necessary for the proper function of the liver (Yuan et al. 2001) in mice.

The number of the EGF repeats in *Delta* proteins varies among species. We discovered nine EGF repeats in the *CapI-Delta* and *Pdu-Delta* sequences comparable to the *Drosophila-Delta* or the *Amphioxus-Delta* (Rasmussen et al. 2007; Vassin et al. 1987). Other *Delta* proteins contain only one (*lag-2 C. elegans*) (Henderson et al. 1994) or six EGF repeats (*Dll3*-mouse) (Dunwoodie et al. 1997). The importance and functionality of these repeats is not yet resolved, but the structure of the EGF regions seems to play an important role in the *Notch*-signaling pathway (Bulman et al. 2000). The EGF repeats of the *Delta* proteins are also being modified by the O-fucosyltransferase 1 and the glycosyltransferase *fringe* (Panin et al. 2002), which influences the *Notch* signaling.

The transmembrane and intracellular region of *Delta* and the DSL-proteins in general are indispensable for the *Notch*-signaling pathway (Hukriede and Fleming 1997; Sun and Artavanis-Tsakonas 1996; Sun and Artavanis-Tsakonas 1997).

The early expression of *CapI-Delta* (St. 4-5) and *Pdu-Delta* (24h) (Fig. 8A1-E; Fig. 9A1-A4) is not comparable. In *Platynereis*, the *Pdu-Delta* transcript is bilaterally located in the proposed larval eye patches in the episphere of the embryo as well as at the posterior edge of the ventral plate. It can be suggested, that these labeled cells of the ventral plate could represent the progenitors of the follicle cell complex including the chaetoblast, which later on forms the chaetal sacs in *P. dumerilii* like in *Nereis vexillosa* (O'Clair and Cloney

1974). In contrast, *CapI-Delta* is expressed in the presumptive segmental tissue in discontinous patches of cells early on. Expression in the CNS and stomodaeum in earlier stages can also be detected in *Capitella* sp. I in contrast to *Pdu-Delta*.

This discrepancy of the *Delta* transcript location in both polychaetes changes during the next stages of development. Both *Delta* genes, *Pdu-Delta* and *CapI-Delta*, are now expressed in and around the earliest forming future chaetal sacs of both embryos. In both animals, the ventral row of expression is stronger than the dorsal one. After a few hours of development in *Capitella* sp. I, the two rows of expression become similar and even more precise in expression, visible as discrete spots. *Platynereis* embryos show *Pdu-Delta* expression in all three chaetal sacs of the ventral row and only two labeled chaetal sacs in the dorsal row. There is also a progressive concentration of *CapI-Delta* transcript from anterior to posterior visible right before the formation of the chaetae, which can not be seen in the *P. dumerilii Delta* gene expression. The latter characteristic of the *Pdu-Delta* and *CapI-Delta* expression is probably due to the different mechanisms of chaetae formation in both polychaetes. *P. dumerilii* forms the three pair of chaetae almost at once in a really short time frame. *Capitella* sp. I chaetae are formed progressively from anterior to posterior in a relative longer developmental process.

CapI-Delta is downregulated right before the start of chaetae formation whereas the *Pdu-Delta* expression is sustained through chaetogenesis and also as all three chaetae pairs appear on each side of the larvae. In comparison to 36h embryos, the *Pdu-Delta* expression is weaker and includes less cells in 48h embryos.

Later on, expression of both *Pdu-Delta* and *CapI-Delta* shifts from the ectodermal to the mesodermal germ layer and becomes restricted to the mesodermal growth zone. This would correspond to the future formation of segments in the juvenile stages of both polychaetes (see Introduction). *CapI-Delta* is also expressed later on in juveniles mainly in the mesoderm with weak expression in the ectoderm. *Pdu-Delta* expression could not be detected in juveniles (see 4.3.2 *Pdu-Notch*).

CapI-Delta is expressed in the CNS and lateral to the mouth in early and also older stages of development. Both expression domains become weaker over time and are not detected in late stages (St. 8-9). In contrast, *Delta* in *P. dumerilii* is not expressed in or around the mouth in early stages, but is in older larvae in the stomodaeum. *Pdu-Delta* also does not show signs of central nervous system expression at any stages of development. It has been

implied that almost all *Delta* genes of different species play a role in neurogenesis (see 4.4.3). The absence of *Pdu-Delta* transcript might imply the existence of at least a second *Delta* gene in *P. dumerilii*.

4.3.2 The receptor *Notch*

The recovered *CapI-Notch* sequence includes more than three complete ankyrin repeats, a PEST domain and an opa repeat. Due to the existence and arrangement of these conserved domains in *Notch* genes (Fleming 1998; Wharton et al. 1985), we conclude that we have isolated a *Notch* gene in *Capitella* sp. I. An almost complete predicted ORF of *Pdu-Notch* is available from Gene-Bank. Though parts of the 3`ORF are missing. The missing sequence is predicted to contain a PEST domain and an opa region (see 3.2.2). However, the available sequence contains enough information to confirm its identity as a *Notch* gene, including 35 EGF repeats, three LNR repeats, an RAM domain and six ankyrin repeats plus a 7th predicted ankyrin repeat.

The number of EGFs in different *Notch* proteins of vertebrates and invertebrates varies significantly from 10 EGFs in *glp-1* (*C. elegans*) (Yochem and Greenwald 1989) to 36 repeats in *Drosophila* (Wharton et al. 1985) or in the human *Notch* homologue *TAN-1* (Ellisen et al. 1991). EGF repeats are indispensable for the binding of the ligands *Delta* or *Serrate* to the receptor *Notch* (Fehon et al. 1990; Fleming 1998). Especially the 11th and 12th repeat of *Drosophila Notch* seems to play a crucial role during the mediation of this interaction (Rebay et al. 1991). Other EGF repeats have also been shown to be important for the interaction of both, *Delta/ Serrate* and *Notch* (Lawrence et al. 2000).

Like *Delta, Notch* is also being modified by the glucosaminyltransferase *fringe* (Moloney et al. 2000; Panin et al. 2002), which shows a preference for certain EGFs to o-fucose (Shao et al. 2003). The ability of *Notch* to bind to its ligands is being altered because of the modification by *fringe* (Shao et al. 2003). The EGF repeats also have even more functions like the homodimerization of *Notch* to increase the response to its ligand or the protection of *Notch* itself against proteases and prevention of ligand-indepent activation of the receptor (Sakamoto et al. 2005).

The six ankyrin repeats are necessary for the binding to *Su(H)* (Greenwald 1994; Lubman et al. 2004; Roehl et al. 1996). The existence of a 7th ankyrin repeat has been proven by the comparison of the homologous consensus sequences in different phyla (Tamura et al.

1995; Wettstein et al. 1997) and by biophysical and biochemical experiments (Zweifel and Barrick 2001; Zweifel et al. 2003). Ankyrin repeats are also called CDC-10 repeats, because these regions were first discovered in the yeast CDC-10/SWI-6 (Reaume et al. 1992). Later on, they were also found in other proteins like NKκB (Kieran et al. 1990) or human erythrocyte ankyrin (Lux et al. 1990).

PEST domains are believed to regulate *Notch* responses by allowing a rapid turnover of the NICD, followed by down regulation of signaling (Kurooka et al. 1998; Oberg et al. 2001). The opa repeat, also part of the *CapI-Notch* gene, was first discovered in the *Drosophila Notch* (Wharton et al. 1985; Wharton et al. 1985) and is characterized by a stretch of several glutamine residues interrupted by histidine residues. The function of this repeat has not yet been reported.

Pdu-Notch and *CapI-Notch* are expressed in three domains of the embryos, the central nervous system, the mouth and the chaetal sacs. In contrast to the limited *CapI-Notch* expression in the belly plates and lateral to the mouth in younger embryos, *Pdu-Notc*h is expressed in a very broad and uniform manner. The early broader expression especially in the ventral plate of *P. dumerilii* could lead to decisions of specific cell fates in this area e.g. between neurogenic and future chaetal sac regions. Thus, the expression in *P. dumerilii* becomes restricted to the CNS, the mouth and the chaetal sacs a few hours later comparable to the *CapI-Notch* expression. Both *Notch* genes are broadly expressed in the presumptive chaetal sac anlagen. The two rows of patchlike *CapI-Notch* expression disappears right before the formation of the chaetae. *Pdu-Notch* expression is sustained through the formation of the chaetae and is still very strong and refined at 48h of development after the completion of the chaetae formation.

The *CapI-Notch* transcript, like *CapI-Delta*, becomes concentrated at the posterior of the larvae over time, where it is maintained as limited expression domain in late stages of embryogenesis. This progressive expression of *CapI-Notch* from anterior to posterior over the successive stages of development can not be observed in *P. dumerilii. Pdu-Notch* expression regions seem to change very abruptly over the course of larval development, which has probably to do with the faster course of embryogenesis in *P. dumerilii*. The expresssion of *CapI-Notch* in the mesodermal growth zone of later embryos can also be seen in *P. dumerilii*, but very weak. Expression of *CapI-Notch* and *Pdu-Notch* in the CNS and in the region of the mouth disappears in later stages of development in both animals.

The ectodermal *Notch* expression in the CNS, mouth and chaetal sacs shifts to the mesodermal growth zone in both *Capitella* sp. I and *P. dumerilii*. Later expression of *Pdu-Notch* as well as *Pdu-Delta* could not be detected in 5d, 7 d and 2-4 week old juveniles in contrast to *CapI-Notch* and *CapI-Delta*. Both transcripts of *Capitella* sp. I were detected in the mesodermal posterior growth zone of juvenile stages and weak expression of both genes in the ectoderm was also observed (E.C. Seaver, unpublished results, see 3.8). Absence of *Pdu-Notch* and *Pdu-Delta* expression in juveniles could have several reasons. First, the protocol for *P. dumerilii* in-situ experiments is not working properly and has to be adjusted even more. The positive juvenile in-situ results for *Pdu-hes1* and *Pdu-Su(H)* would argue against this explanation (3.2.3). Secondly, there is in fact no expression of *Pdu-Delta* and *Pdu-Notch* in later stages in *P. dumerilii*. This explanation is very unlikely, because of the involvement of the *Notch* signaling pathway in almost all developmental processes especially during and shortly after embryogenesis and larval development (Artavanis-Tsakonas et al. 1995; Bray 1998; Hansson et al. 2004; Lai 2004).

4.3.3 The *hes*-family

Five *Capitella*-bHLH-O sequences and two *P. dumerilii*-bHLH-O genes were isolated or found by searching through the trace files of the sequenced *Capitella* sp. I genome. To classify the sequences within this group of genes, phylogenetic analyses were performed. *CapI-hesr1* and *CapI-hesr2* were clearly placed into the group of *hesr/hey* genes in all three consensus trees. These are the first two isolated *hesr/hey* genes of a lophotrochozoan and two of the first invertebrate *hesr*-genes besides the *Drosophila hesr-1* gene (Kokubo et al. 1999). The amino acid sequence also shows the characteristic glycine residue right at the beginning of the bHLH domain (Davis and Turner 2001).

The *CapI-hes2* and *CapI-hes3* sequences were placed together with the *Enhancer of split* genes of *Drosophila* as a separate invertebrate *hes* group independently from the vertebrate *Enhancer of split* group.

The definite classification of the remaining three genes, *Pdu-hes1, Pdu-hes2* and *CapI-hes1,* is more complicated and obviously not possible with the analyses used. All other sequences of different species and bHLH-O groups used for the calculation were expected results from compared to the phylogenetic analysis done by Davis and Turner (1997). With a Bayesian analysis, the *Pdu-hes1* gene is classified as an *Enhancer of split* gene. *Pdu-hes2*

and *CapI-hes1* are placed at the base of the *hairy* genes and invertebrate *Enhancer of split* genes. The Neighbor-Joining tree shows the classification of the latter two sequences into the *hairy* family, but also *CapI-hes2* is grouped into this gene family instead of into the *Enhancer of split/ hes* family. The comparison of the expression patterns of all five genes (*CapI-hes1, 2, 3* and *Pdu-hes1, 2*) is consistent with the Bayesian analysis by far. These analyses were performed to classify the isolated *hes* sequences into the group of bHLH-O transcription factors and inside one or two of the four subfamilies. No further predictions can be made as towards the relationship of animal groups.

Pdu-hes1, *CapI-hes2* and *CapI-hes3* show expression in or around the presumptive chaetal sac anlagen (for *CapI-hes*2 and *CapI-hes3*, E.C Seaver, unpublished results). After being expressed very uniformly in an early stage of development, *Pdu-hes1* expression matures into a pattern surrounding the presumptive chaetal sac anlagen. Later on, it is expressed in the mesoderm of the posterior growth zone comparable to *CapI-hes1* (see 4.2). The expression in the head at 4d could be associated with the development of palps and their sensory cells.

Pdu-hes2, in contrast to *Pdu-hes1*, is not expressed around the chaetal sacs or in the terminal growth zone. The only similarity in the expression of both genes seems to be the transcript localization in the mouth, although *Pdu-hes1* is expressed around the mouth and *Pdu-hes2* shows expression towards and in the stomodaeum of the embryo. All expression domains of *Pdu-hes2* seem to be ectodermal, including the staining of the stomodaeum which persists throughout all stages of development or the labeling of the vetral midline cells. This very weak expression of *Pdu-hes2* was confirmed by several in-situ experiments and it can be detected from 36h to 60h of development. *Pdu-hes2* expression in the middle of the ventral plates is similar to the reported expression of *Pdu-slit* and *Pdu-netrin* in the early differentiating midline at the same stage of development (Denes et al. 2007). The two patches of expression posterior of the mouth could coincide with the described pigmented area (Wilson 1892), but also anlagen for the anal cirri later on. Expression of *Pdu-hes2* consisting of four bilateral patches in the episphere of the embryo could be part of the developing CNS or special sensory organs.

The *CapI-hes1* transcript is located in the mesoderm from the beginning of its expression. The gene is expressed in a striped like pattern in the unsegmented part of the belly plates and later on in the posterior growth zone. With its expression pattern, it clearly follows the proposed two-phase segmentation process of *Capitella* sp. I (see Introduction) (Seaver et

al. 2005). First, nine to ten segments are formed by the lateral segment precursors and then the segmentation process is continued by the terminal growth zone. It can be proposed, judging from its expression pattern in the unsegmented lateral tissue and later in the posterior growth zone, that *CapI-hes1* is involved in the segmentation process of *Capitella* sp. I (Fig. 15G-I). Strikingly, *CapI-hes2* and *CapI-hes3* expression in juveniles coincide with *CapI-hes1* expression in the mesoderm of the posterior growth zone even though expression of *CapI-hes2* and *CapI-hes3* is broader and not as restricted as *CapI-hes1* expression (Fig. 26J-P). All three genes are probably involved in segment formation during juvenile development in *Capitella* sp. I, but on different levels during the segmentation process like proliferation and growth of segments.

The comparison of the isolated *hes* genes to each other and of the two polychaetes plus their expression is very complex and quite difficult. One explanation might be the existence of several additional members of the bHLH-O gene family in one of the two species or both species. We can not determine if the isolated *hes /hesr* genes are orthologues of each other. The possibility that even more bHLH-O genes are existent in the genome of both polychaetes, especially in *P. dumerilii*, is very high. In all studied model organism like *Drosophila, Xenopus* or chicken, there are several members of the *hes/hey/hairy* family (Davis and Turner 2001; Kageyama et al. 2007). For example in the *Danio* genome, 15 *hes* genes (called *her*-genes for *hairy* and *E(spl) r*elated genes) exist and for mouse 7 *hes* genes were isolated so far (Sieger et al. 2004). In *Drosophila*, the *deadpan* and *hairy* transcription factors were identified as ‚true' *hairy* genes (Bier et al. 1992; Carroll et al. 1988; Younger-Shepherd et al. 1992). Besides those sequences, other members of the bHLH-O group were also isolated in *Drosophila* like a *hers*-gene or the *Enhancer of split* complex (Knust et al. 1987; Kokubo et al. 1999). This does not include the *hey* and *hesr* sequences of these model organisms. All of the already studied *hes/her* genes have different functions and are involved in many different developmental processes (Bessho et al. 2001; Henry et al. 2002; Kageyama et al. 2007; Shankaran et al. 2007). Therefore, it is also not too surprising, that *CapI-hes1, Pdu-hes1* and *Pdu-hes2* show completely different expression patterns. Functional studies could probably confirm the different functionalities of the *hes* genes in *Capitella* sp. I and *P. dumerilii*.

4.4 Comparative analysis of the *Notch* signaling pathway in *Capitella* sp. I and *P. dumerilii*

4.4.1 General comparison

Capitella sp. I and *P. dumerilii* are two very diverse members of the polychaete family. Their life histories are completely different from each other (see Introduction). Thus, in some cases, it is very hard to compare the expression patterns of different genes in these two organisms.

In both annelids, the *Notch* gene is always expressed in a broader region than *Delta. Notch* resembles the expression of *Delta* and it is expressed in the same domains. Thus, the overlapping expression of *Notch* and *Delta* in both polychaetes emphasizes the high possibility of a ligand/receptor relationship between both genes. In general, the *Notch* gene is expressed broadly in younger stages in both organisms. Its expression becomes more restricted at later stages. This refinement is particularly apparent for *Pdu-Notch* from 24h to 48h of development.

Besides the good evidence of a second *Delta* gene in *P. dumerilii* (see 4.2), there is also the possibility of another *Notch*-DSL-ligand called *Serrate/Jagged* (Katsube and Sakamoto 2005) in both polychaetes (see 4.1 for *fringe*). This ligand is only called *Jagged* in mammals (Lindsell et al. 1995). Like *Delta*, the alternative ligand of *Notch*, *Serrate* is involved in multiple processes of development (Irvine and Vogt 1997; Portin 2002). It has been found in many vertebrates studied so far like *Xenopus*, zebrafish, chicken and mouse, but also *Drosophila* and *Ciona intestinalis* (Kiyota and Kinoshita 2002; Lissemore and Starmer 1999; Satou et al. 2003).

An overlap of *Pdu-Notch* expression and its possible downstream targets *Pdu-hes1* and *Pdu-hes2* is visible in 24h stages. *CapI-hes1* shows almost no overlap with *CapI-Notch.* These facts would acount for a second *Notch* gene in both polychaetes or for a *Notch* independent *hes*-pathway (see also 4.1, 4.2, 4.3.3). Expression of *CapI-hesr1* and *CapI-hesr2* was not studied. It would be very interesting to see wether the expression patterns are part of the canonical *Notch* pathway. *Hesr/hey* genes have not yet been observed in detail either in vertebrates nor invertebrates, but it is known that they are involved in the heart and vessel development as well as in gliogenesis (Kokubo et al. 2005; Kokubo et al. 2005; Kokubo et al. 2004) of *Drosophila*, *Xenopus*, zebrafish and mouse (Rutenberg et al.

2006; Taelman et al. 2004; Zhong et al. 2001). *Hesr-1* expression in *Drosophila* also depends on *Notch* signaling (Kokubo et al. 1999).

Even though most of the genes were expressed around and/or in the stomodaeum in both polychaetes, the patterns seem very variable in this region and and can not be interpreted easily. *CapI-Delta, CapI-Notch* and *CapI-fringe* expression coincides in two domains lateral to the mouth. It might be associated with the developing foregut, but also with the circumesophageal or suboesophageal nervous system. *Pdu-Notch, Pdu-hes1* and *Pdu-hes2* seem to be involved in the development of the stomodaeum, because they are expressed very early around the mouth and later in the stomodaeum. It is also possible, that they are involved in the formation of esophageal nerves. *Pdu-hes1* expression of 36h to 72h of development is possibly associated with the muscles of the mouth, because the transcript is located in deeper layers of the embryo and around but not in the stomodaeum. Maybe it is also associated with the formation of the forgut. Later expression of *Pdu-Notch* and *Pdu-Delta* (60h and 72h) seems to be more associated with the circumesophageal nervous system based on the precise localization of both transcripts in spots around the stomodaeum. Later expression of *Pdu-Su(H)* and *Pdu-hes2* is very broad in and around the stomodaeum possibly associated with the developing forgut.

4.4.2 The *Notch* signaling pathway and chaetogenesis

As mentioned previously (see 3.1.1), chaetae are formed by a chaetoblast and the surrounding follicle cells. The chaetoblast gives rise to different microvilli. On the surface of these microvilli secreted chaetal material of the chaetoblast and the follicle cells assemble forming the future chaetae and due to the central microvillus in each of them turns them into hollow structures. Biochemically, it has been assumed that N-acetyl-glucosamine monomers and β-chitin chains are being linked by proteins with the help of enzymes to form a chaetae, but this has not been proven by biochemical experiments and analyses (Hausen 2005). Development and anatomy of chaetae is comparable across polychaetes, clitellates and echiurids (Hausen 2005). Chaetogenesis of *Capitella* sp. I was described in detail by Schweigkofler et al. (Schweigkofler et al. 1998), whereas the chaetogenesis of Nereids was described by Gustus and Cloney (Gustus and Cloney 1973).

Strikingly, the involvement of the *Notch* signaling pathway during chaetogenesis in both polychaetes seems very apparent. *Pdu-Delta, Pdu-Notch, CapI-fringe, CapI-Notch* and *CapI-Delta* show expression patterns consistent with this proposed function.

CapI-Delta and *CapI-Notch* expression disappears right before the formation of chaetae, while *Pdu-Delta* and *Pdu-Notch* are still expressed after chaetae have been formed. The expression of these *P. dumerilii* genes is no longer anymore around 60h of development. It can be proposed that this later disappearance of gene expression correlates to the rapid larval development of *P. dumerilii* in comparison to the comparatively long embryogenesis of *Capitella* sp. I.

Surprisingly, the gene *CapI-hes2* shows very restricted, larval expression in the presumptive chaetal sacs (E.C. Seaver, unpublished results) in contrast to the expression of *CapI-hes1, Pdu-hes1* and *Pdu-hes2*. Thus, the expression patterns of *CapI-hes2, CapI-Notch* and also *CapI-Delta* indicate an involvement of all three genes in the chaetogenesis of *Capitella* sp. I, as do *Pdu-Delta* and *Pdu-Notch* in *P. dumerilii*.

Even though only a few components of the canonical *Notch* pathway were isolated from *Capitella* sp. I and *P. dumerilii*, one can speculate about the molecular mechanism behind the formation of chaetae in both polychaetes. Since chaetogenesis is similar in all annelids (Hausen 2005), we can assume a common molecular mechanism. Two developmental processes are typical for *Notch* signaling: lateral inhibition and lateral induction including lineage decision and boundary formation (see Introduction) (Bray 1998).

Lateral inhibition by *Notch*-signaling is a central component of several different developmental processes such as oogenesis in *Drosophila* (Ruohola et al. 1991), vulva development in *C. elegans* (Newman et al. 1995; Sternberg 1988), myogenesis (Bate et al. 1993) or neurogenesis in invertebrates and vertebrates (Fortini and Artavanis-Tsakonas 1993; Lewis 1996; Xu et al. 1990). One of the best studied examples is certainly neurogenesis in *Drosophila*. The central nervous system of the fruit fly arises from neuroblasts. They segregate individually from a so called neurogenic region, enlarge and move internally, where they form the CNS (Hartenstein and Campos-Ortega 1984). The neurogenic region contains equipotent cells, which can differentiate into either neuroblasts or epidermal cells, which surround the neuroblasts. Before the segregation of neuroblasts sets in, proneural genes are expressed in a repetitive pattern of small cell clusters in the proneurogenic region (Martin-Bermudo et al. 1995; Martin-Bermudo et al. 1991; Skeath

and Carroll 1992). The spacing of the clusters is accomplished by lateral inhibition: In *Notch* and *Delta* mutants, proneural genes are expressed uniformly since no lateral inhibition occurs (Cabrera 1990; Skeath and Carroll 1991). In *Notch* and *Delta* mutants, proneural genes are expressed uniformly and no lateral inhibition occurs (Cabrera 1990; Skeath and Carroll 1991). This means, that the *Notch* signaling pathway is responsible for the silencing of proneural genes in cells that are supposed to become epidermoblasts and not neuroblasts. This silencing is achieved by the expression of bHLH genes induced by *Notch* (Dawson et al. 1995). The single cell, which is supposed to become the neuroblast, is expressing *Delta* in high levels and therefore adopts the primary cell fate to become a neuroblast. This cell inhibits its neighbors to become a neuroblast by activating *Notch*, which then promotes the differentiation of the surrounding cells into epidermoblasts. This process is called lateral inhibition (Portin 2002). There are also other factors, which must be taken in account in such a complex processes like the lateral inhibition e.g. the evidence of possible cross-talk between *Notch* and other pathways like EGF or *wingless* (Couso and Martinez Arias 1994; Price et al. 1997), but this will not be discussed in detail in this work.

Lateral inhibition results in a so called “salt and pepper” pattern of gene expression (Lewis 1996; Lewis 1998).in which *Notch* is activated as described above. This activation of Notch also serves as an inhibition of the *Notch* ligand production. The cell that produces more ligand (neuroblast) forces its neighbors (epidermal cells) to produce less ligand. This inhibition is the reason for the differential expression pattern one finds in a restricted field. Assuming this mechanism for chaetogenesis in polychaetes, the early discontinous *CapI-Delta* expression in the belly plates can be explained. The broader expression of *CapI-Notch* favors this hypothesis. Later expression of *Pdu-Notch* and *Pdu-Delta* in the presumptive chaetal sacs display the same restricted pattern, which probably supports this theory of lateral inhibition. Earlier expression before 24h of development was not studied in *P. dumerilii*, but might show the same course of development like in *Capitella* sp. I.

From the expression patterns, the second possible mechanism of *Notch* signaling, lateral induction, is not favoured in the context of chaetogenesis since this mechanism typically produces sharp boundaries of expression. Additionally, cells would be expected to have similar expression patterns and the ligand would be expressed locally uniform. This cannot be observed in the *CapI-Delta* “salt and pepper” pattern (Cornell and Eisen 2005; Lewis 1998). Lateral induction plays an important role in e.g. limb development (de Celis et al. 1998; Rauskolb 2001), wing development (de Celis and Bray 1997; Rauskolb et al. 1999)

and probably also during somitogenesis (Gossler and Hrabe de Angelis 1998). The characteristic expression of the ligand is substantiated by the activation of *Notch*, which promotes the production of the ligand. If cells express a high level of the ligand, it stimulates its neighbor to do the same and therefore adopt the same cell fate. This all-or-nothing behavior leads to the formation of sharp boundaries like in the wing of *Drosophila* (Panin et al. 1997). This is not the case for *Delta* and *Notch* expression in neither in *Capitella* sp. I nor in *P. dumerilii*.

4.4.3 Neurogenesis

Notch plays an indispensable role in the development of the central and also peripheral nervous system in almost all studied organisms so far, vertebrates as well as invertebrates (Beatus and Lendahl 1998; Fortini and Artavanis-Tsakonas 1993; Lewis 1996). However, the nervous system of *Hydra vulgaris* forms an exception. An effect in neurogenesis after the treatment with the reagent DAPT (see below) could not be detected in that species (Kasbauer et al. 2006). In other animals, *Notch* signaling plays a key role in neurogenesis and controls lateral inhibition as described above (4.4.2) and was believed to control asymmetric cell division.

The latter mechanism however is independent of ligand-expression in the *Notch* signaling (Bray 1998). During bristle and muscle development in *Drosophila melanogaster* asymmetrical distribution of a cytoplasmic regulator between the daughter cells control determination and differentiation (Roegiers and Jan 2004; Ruiz Gomez and Bate 1997). *Notch* is required for decisions in certain cell lineages by being activated in one cell but not the other (Knoblich 1997; Roegiers and Jan 2004). The cytoplasmic regulator, called *Numb* in this case, is inherited unequally by the daughter cells (Knoblich et al. 1995; Rhyu et al. 1994). *Numb* antagonizes *Notch*, so that only one cell has activated *Notch* signaling. Like in the process of lateral inhibition, asymmetric cell decisions also involve more than just two components and are influenced by multiple factors (Gho and Schweisguth 1998; Knoblich 1997; Roegiers and Jan 2004). This molecular system has been found in invertebrates as well as in vertebrates (Kimble and Simpson 1997; Petersen et al. 2006; Weller and Tautz 2003; Zhong et al. 1997). It would be very intriguing to find and analyze the involvment of *Numb* or other regulators of the *Notch* signaling pathway in neurogenesis or other developmental processes in the two annelids. Perhaps, the nervous

system in *Capitella* sp. I or *P. dumerilii* is being patterned by lateral inhibition, asymmetric cell division or even lateral induction.

Almost all genes studied, except for *Pdu-Delta* and *CapI-hes1*, show expression domains consistent with their involvement in the development of the CNS. Even *Pdu-mef2* is transiently expressed in the upper epishere at 24h. The *Capitella* sp. I genes show expression in the cerebral ganglia and some also in the proposed circumpharyngeal nervous system. This expression weakens over time and disappears in late larval stages. *P. dumerilii* genes are also expressed in the central nervous system. *Pdu-Su(H)* even shows expression in the brain at juvenile stages of development. This late expression of *Pdu-Su(H)*, the expression of *Pdu-hes2* in the differentiating midline (36h-60h, Fig. 19C1-E1, long arrow) and *CapI-fringe* expression in the ventral neuroectoderm (Fig. 21C, D1 and E1, long arrows) do not coincide with the transcript of either *Notch* genes, which suggests the presence of a second *Notch* gene in both organisms, *Capitella sp. I and P. dumerilii*. The possibility of *Notch*-independent processes can not be ruled out, but has not yet been reported for these genes in the context of neurogenesis. *Su(H)* has been proven to play a crucial role in neurogenesis of vertebrates and invertebrates (Fortini and Artavanis-Tsakonas 1994; Lecourtois and Schweisguth 1997; Oka et al. 1995; Schweisguth 1995; Wettstein et al. 1997), therefore it is not too surprising that the expression of *Pdu-Su(H)* is also located in the nervous system.

Functional studies would be very helpful to interpret the expression patterns of the genes. An RNAi protocol has not yet been established for *Capitella* sp. I. In *P. dumerilii*, the protocol is not reliable i.e. it does not work for all genes. Recently, we found a more reliable and very fast method for inhibiting the *Notch* signaling pathway by using the reagent DAPT mentioned above for *Hydra vulgaris* (Kasbauer et al. 2006). DAPT (N-[N-(3,5-diflourophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester) is supposed to inhibit the γ-secretase complex including presenilin (see Introduction). This enzyme normally cleaves the *Notch* receptor besides other components resulting in release of the NICD activating the transcription of genes. It has been shown in the past, that the application of DAPT in *Drosophila*, zebrafish or also *Hydra* results in developmental defects, which correspond to *Notch* mutations (Geling et al. 2002; Kasbauer et al. 2006; Micchelli et al. 2003). Application of the drug resulted in neurogenic defects in zebrafish and *Drosophila*. Surprisingly, *Hydra* does not show any defects in the formation of neuroblasts and differentiation of nerve cells, but in the development of oocyte precursor cells and

nematocytes (Kasbauer et al. 2006), comparable to a role in oocyte production in *Drosophila* and zebrafish.

Preliminary results of the inhibition of the *Notch* signaling pathway in *P. dumerilii* are also available now (Fuchs et al. in prep.). Up to now, we are not able to prove the complete inhibition of *presenilin* directly and thus the inhibition of the *Notch* signaling pathway like Käsbauer et al. (2007) did in *Hydra*, but we can assume the same effect for the application of DAPT to *P. dumerilii* embryos like in *Danio, Drosophila* or *Hydra* due to the proposed conservation of the *Notch* signaling across phyla (see Conclusion). The application of the drug in a concentration of 20µM and also 40 µM showed clear effects on the development of the *P. dumerilii* embryos, which are consistent with an inhibition of the *Notch* signaling pathway by DAPT. Neurogenesis in the brain as well as in the ventral nerve cord was severely affected. The CNS was in general very disorganized. Chaetae were not developed, which would support the expression of *Pdu-Delta*. Even myogenesis seemed to be influenced by the inhibition, because certain groups of muscles were not formed properly. Later on in development, eyes and jaws were not formed. Effects on the development of the stomodaeum and foregut were not studied. The application of DAPT to the embryos has probably also even more effects. In-situ experiments with DAPT treated embryos for *Pdu-Delta, Pdu-Notch* and also proposed downstream targets like *Pdu-hes1* or *Pdu-hes2* are still outstanding studies. We still have to prove the inihibition of presenilin and the nuclear translocation of the NICD indirectly or directly. With these DAPT experiments, we are beginning to understand the immense involvement of *Notch* and its components during the development of *P. dumerilii*. Similar experiments would also give necessary insights into the role of the *Notch* signaling pathway during the development of *Capitella* sp. I.

4.4.4 The *Notch* signaling pathway and its involvement in the segmentation process

Early larval expression of all genes in *P. dumerilii* and *Capitella* sp. I studied in this project except for *CapI-hes1* do not show a typical striped like pattern in the growth zone or in the future segmented tissue as observed in arthopods like chelicerates, myriapods or insects (Dearden and Akam 2000; Dove and Stollewerk 2003; Kadner and Stollewerk 2004; Schoppmeier and Damen 2005; Stollewerk et al. 2003). Expression *Pdu-Notch* genes and its components in *P. dumerilii* does also not resemble the expression patterns during somitogenesis in vertebrates (Jiang et al. 2000; Rida et al. 2004). *CapI-Notch* with its

broad expression in the belly plates is more comparable to the expression domains analyzed in vertebrates.

In animals where *Delta* is involved in segment formation, it is expressed in the posterior growth zone and anteriorly in a striped like pattern in the posterior part of the newly formed segments. This can be seen in vertebrates as well as in invertebrates (Jen et al. 1997; Kadner and Stollewerk 2004; Palmeirim et al. 1998; Stollewerk et al. 2003), but not in the early expression patterns of *Pdu-Delta* or *CapI-Delta*. In addition, some *Delta* orthologues show a cyclic expression pattern prior to somite formation in the presomitic mesoderm during vertebrate somitogenesis and in a dynamic pattern in the ectodermal growth zone before and during segmentation of chelicerates (Jiang et al. 2000; Jiang et al. 1998; Rida et al. 2004; Stollewerk et al. 2003). No dynamic expression of *CapI-Delta* or *Pdu-Delta* could be observed.

Fringe is usually expressed in the posterior growth zone and transiently in the newly formed segments in vertebrates and invertebrates (Dearden and Akam 2000; Evrard et al. 1998; Johnston et al. 1997; Prince et al. 2001; Sakamoto et al. 1997), not so in *Capitella* sp. I.. Cyclic expression of *fringe* is crucial during vertebrate somitogenesis (Aulehla and Johnson 1999; Serth et al. 2003) and affects *Delta* and *hes* transcription. Dynamic expression of *CapI-fringe* was not observed, but studies and analyses of *CapI-fringe* expression in St.8-9 larvae and juveniles are missing.

Hairy and *Enhancer of split* genes are always expressed in a striped like manner if involved in segmentation and somitogenesis (Bessho et al. 2001; Palmeirim et al. 1997). *Pdu-hes1* and *Pdu-hes2* (as well as *CapI-hes2* and *CapI-hes3*) do not show an early expression pattern corresponding to a possible involvement in segmentation like an expression in stripes. In contrast to that stands the *CapI-hes1* expression with its segmentally iterated expression pattern in the unsegmented part of the belly plates. Strikingly, the expression of *CapI-hes1* is very localized in the lateral domain and does not span the medial/lateral width of each segment (Davis and Patel 2003; Rida et al. 2004). If *CapI-hes1* is really involved in segmentation, it is unclear how it can act over such a distance to organize segment formation and differentiation. One possibility would be the different location of *CapI-hes1* RNA and its protein. Antibody staining for this specific *hes*-gene would localize the *CapI-hes1* protein, so it might be more obvious, how the protein itself can act. In mice, the oscillation of *hes*-transcription is connected to the degradation of its protein Hes-1 and thus a negative, cell autonomous autoregulation of

both transcript and protein occurs (Hirata et al. 2002). This could also be the case for *CapI-hes1* and its protein.

Hes genes and their transcription are also regulated by the *Notch* signaling pathway in vertebrates (Bessho et al. 2001; Jouve et al. 2000) and as shown in recent results also in basal arthropods like the spider *Cupiennius salei* (Stollewerk et al. 2003). When knocking out *Cs-Notch, hairy* expression was also disturbed in the spider. The experiment provides an evidence for the *Cs-hairy* gene being a downstream target of the *Notch* signaling pathway in spiders. In contrast, *Drosophila* segmentation is not under the influence of *Notch* and its components, which has probably something to do with the different segmentation modes in both organisms (see Introduction). *CapI-hes1*, which is only expressed in the presumptive segmental tissue and the posterior growth zone throughout the stages of *Capitella* sp. I development, could be regulated via a second *Notch* or a completely *Notch*-independent pathway, even though we can assume, that the control of *hairy/hes* by *Notch* in the context of segmentation is an ancestral feature of arthropods (Patel 2003; Peel 2004; Peel et al. 2005). On the other hand, it may not necessarily be the case in polychaetes.

The early weak uniform expression of *Pdu-Su(H)* shows overlapping expression pattern with *Pdu-Notch, Pdu-Delta* or the *hes* genes. In juveniles, *Pdu-Su(H)* showed expression domains in the brain, but not in any other regions including the posterior growth zone. It can be assumed, that a second *Su(H)* gene exists in *P. dumerilii* with a proposed overlapping expression to *Pdu-Notch* or other components of the signaling pathway. In other organisms, *Su(H)* orthologues are expressed more or less uniformly, which would fit with the early expression patterns of *Pdu-Su(H)* (see 4.2) (Furukawa et al. 1995; Sieger et al. 2003). Their function is indispensible for somitogenesis in vertebrates and also segmentation in invertebrates (Schoppmeier and Damen 2005). Mutations of *Su(H)* genes in both animal groups show even more severe phenotypes than *Notch* mutants but comparable to those, which shows the conservation of *Su(H)* in the *Notch* signaling pathway in both, invertebrates and vertebrates (Furukawa et al. 1995; Lecourtois and Schweisguth 1997; Sieger et al. 2003; Wettstein et al. 1997).

Several years ago, a pair-rule like pattern of *her1* in vertebrates was suggested comparable to insects and other arthropods (Muller et al. 1996), but this paradigm was resolved and a segmental expression pattern was also demonstrated similar to what has been documented in chicken (Holley et al. 2000; Palmeirim et al. 1997). These misinterpreted results brought

back the discussion of the possibility of a common segmented ancestor in Bilaterians (see Introduction). The results of Stollewerk and collegues (2003) support the theory. Our results in the two studied polychaetes, *Capitella* sp. I and *P. dumerilii*, show that there is no evidence for the involvement of the *Notch* signaling pathway in the larval segment formation of those two annelids except for the expression of *Pdu-Notch* and *Pdu-hes1* at 24h. Both genes display very broad expression early on, therefore an involvement in the process of segmentation at this stage can not be excluded (see Fig. 12A1-4, Fig. 18A1-4). We will have to study even earlier stages to determine the localization of both transcripts in i.e. the forming ventral plate. It is also possible, that the *Notch* signaling at this stage in *P. dumerilii* has an influence in the further development of the cells in the ventral plate.

Late larval expression of all genes in *Capitella* sp. I and *P. dumerilii* is located in the mesoderm and partly in the ectoderm of the posterior growth zone. Expression in this region could mean a possible involvement of those genes in the addition of segments from the posterior growth zone. The postulated two phase segmentation mode in *Capitella* sp. I (Seaver et al. 2005) would also fit in this theory as well as the juvenile expression later on. Juvenile expression patterns especially in *Capitella* sp. I might support a role of the *Notch* signaling pathway in the terminal addition of segments in polychaetes. Expression of the main components *CapI-Notch, CapI-Delta, CapI-hes1, hes2* and *hes3* (Fig. 26) as well as *Pdu-hes1* in the mesoderm of juveniles and for some also in the ectoderm of the posterior growth zone and last formed segment would mean an involvement of these genes in the segmentation process of *Capitella* sp. and *P. dumerilii. CapI-Notch, CapI-Delta, CapI-hes2* and *CapI-hes3* coincide in their expression in the nascent segment. Thus, it is possible, that *Notch* and *Delta* are regulating those two *hes* genes during juvenile growth of *Capitella* sp. I. The regulation of *CapI-hes1*, which is only expressed in the posterior growth zone, but not in the nascent segment in contrast to *CapI-Notch* and *CapI-Delta*, might occur via the transciption and/or proteins of *CapI-hes2* and *CapI-hes3*. It is also possible that the protein of *CapI-hes1* is migrating into the nascent segment to interact with *CapI-Notch*. In both annelids, no arthropod like expression of any gene studied in this thesis was detected in contrast to *Pdu-en* or *Pdu-wg* (Prud'homme et al. 2003). Other expression patterns of components of the *Notch* pathway in *P. dumerilii* (*Pdu-Notch, Pdu-Delta, Pdu-Su(H)* and *Pdu-hes2*) were not detected in juveniles, but technical problems with the in-situ protocol or fixation of the young worms can not be excluded.

4.4 The *Notch* signaling pathway and the muscle marker *Pdu-mef2*

All expression domains of *Pdu-mef2* at 24h are located in the ectoderm. At 36h hours of development, *Pdu-mef2* expression has changed and can only be found in the mesoderm around the presumptive chaetal sacs. Not a single ectodermal expression domain of the 24h embryos is sustained through this stage. Expression of *Pdu-mef2* in 36h embryos of *P. dumerilii* shows strinking similarity to the expression pattern of *Pdu-hes1* at the same stage of development (compare Fig. 18C2 and 3 and Fig. 24B2-3 and C). This fact, besides the microscopic analysis of the transcript in deeper layers of the embryo, would also account for the mesodermal expression of *Pdu-hes1* at this stage and later on.

The *Notch* signaling pathway is involved in multiple processes of development including somitogenesis (see above and Introduction) and myogenesis (Luo et al. 2005). *Notch* influences the differentiation and development of muscles via the activation of *hes* genes expression (Cossins et al. 2002; Gao et al. 2001), which are necessary for myogenesis and activate the development of muscles. So it is not too surprising, that one *hes* gene *Pdu-hes1* and its expression is overlapping with the transcript of *Pdu-mef2*. *Notch* can also inhibit two main factors for myogenesis, MyoD and also mef2C (Wilson-Rawls et al. 1999).

Mef2 genes and especially *hes*-genes are usually connected to the *Notch* pathway. The mesodermal expression of both genes, *Pdu-hes1* and *Pdu-mef2*, would also account for the existence of a second *Notch* gene in *P. dumerilii*, because *Pdu-Notch* is not expressed in the mesoderm at that time of development. We also cannot rule out a *Notch*-independent mechanism for the expression and function of *Pdu-mef2* and *Pdu-hes2* in *P. dumerilii*.

4.5 Conclusions

Even though the life histories and the development of both polychaetes *Capitella* sp. I (Capitellida) and *Platynereis dumerilii* (Phylodocida) possess tremendous differences, similarities in the expression patterns of several genes are striking. These congruities indicate a common functionality of the *Notch* signaling pathway and its components in certain processes like chaetogenesis, neurogenesis and the development of the stomodaeum and possible in the foregut.

With the overlapping and coinciding expression patterns of the genes studied and presented in this dissertation, we can assume a conservation of the canonical *Notch* signaling pathway between *Capitella* sp. I and *P. dumerilii*. The isolated receptors *Pdu-Notch* and *CapI-Notch* as well as their proposed ligands *Pdu-Delta* and *CapI-Delta* have expression patterns which are good evidence for a receptor-ligand relationship of *Notch* and *Delta* in both polychaetes. *CapI-fringe* displays with its expression pattern a good candidate to regulate *CapI-Notch* in *Capitella* sp. I. *CapI-hes1* might be regulated by *Notch* later on in juvenile growth even though a larval regulation is not probable. *CapI-hes2* and *CapI-hes3* support the hypothesis of a downstream target of *CapI-Notch* in the larval as well as in the juvenile growth. Also, the experiments with DAPT inhibiting the γ-secretase and the transport of NICD in the nucleus support the role of the *Notch* signaling pathway in the nervous system, chaetoblasts and several other organs as well. This accounts for high conservation of the mechanism of the *Notch* signaling pathway and its components across phyla in general.

We do not assume an involvement of the mechanism of asymmetric cell division (see 4.4.3) in the process of chaetogenesis, because expression of the proposed involved components (*Pdu-Notch, Pdu-Delta, CapI-Delta, CapI-Notch* and *CapI-fringe*) show expression which resembles the process of lateral inhibition, even though the other processes of asymmetric cell division and lateral induction can not be excluded. Indeed, there may be completely alternative mechanisms of *Notch*-signaling in *Capitella* sp. I and *P. dumerilii*.

Several features of the genes studied are inconsistent and provide evidence for an additional *Notch* gene in *Capitella* sp. I and *P. dumerilii* (as was already shown for many other animal taxa): A) Functional experiments in *P. dumerilii* with the *Notch* inhibitor DAPT (Fuchs et al., in prep.) showed severe neurogenic phenotypes especially in the

ventral nerve cord even though *Pdu-Notch* is not specifically expressed there. Genes involved in the formation of the ventral neuroectoderm and later on of the ventral nerve cord in *P. dumerilii* are expressed like listed in the paper of Denes and colleagues (Denes et al. 2007) also in later stages than 24 or 36h of development.

B) *Pdu-hes2* is expressed in the ventral midline probably overlapping with the conserved midline repellent *Pdu-slit* (Kidd et al. 1999) and the gene *Pdu-netrin,* which is usually expressed in the midline of different bilaterians (Serafini et al. 1994; Shimeld 2000).However, *Pdu-Notch* expression can not be found in that region.

C) *CapI-fringe* expression also shows expression in the developing ventral nerve cord without *CapI-Notch* expression. *Fringe* is an enzyme and regulator of the *Notch* pathway. It has not yet been reported, that it acts without *Notch* or one of its ligands.

D) *CapI-hes1* and *Pdu-hes1* show expression exclusively in the mesoderm. Both genes have to be regulated somehow, but the weak overlap of expression, if any, with the *Notch* genes, does not seem to be equivilent to regulate the *hes1* genes of both polychaetes. The same assumption can be made for *Pdu-mef2* and *Pdu-Notch* as mentioned above.

By searching through the trace files, preliminary results do show a proposed second *Notch* gene in the genome of *Capitella* sp. I (E.C. Seaver, unpublished results), which supports our assumption. Of course, we can not completely exclude, that certain expression patterns might have adopted *Notch*-independent pathways. Still, we also predict the existence of at least a second *Pdu-Delta* and *Pdu-Su(H).*

Addition of segments in polychaetes after embryonic development can be seen as an ancestral feature for this phylum as well as for many lower arthropods. How this formation of segments is being established on the molecular level is still not resolved. There are many candidate genes, which have been studied and have to be studied in the future to find out what common mechanisms might be involved in these processes. The *Notch* signaling pathway and its components are good candidates for being involved in the posterior addition of segments even though a wider sampling in the group of annelida is necessary to determine the ancestry of the involvement of *Notch* in the segmtenation of polychaetes or even annelids.

To resolve the question of the common segmented or unsegmented ancestor, more research and sampling has to be done from present as well as extinct species. Both scenarios of a convergence of segmentation in different groups as well as a segmented urbilaterian ancestor are possible. Conserved pathways across phyla like wnt, FGF or even *Notch* are

involved in almost all developmental processes, so it would not too surprising if *Notch* plays a role in segmentation across all animal groups like it does in neurogenesis, myogenesis, appendage development, oogenesis or immunology. The big question is if segmentation was developed by convergence or if it displays a common ancestral feature across all phyla. Of course, we could not resolve this problem with the results of this work, but we can add another puzzle to the big picture established in the last decades of research and also emerging within the next couple of years.

4.6 Future directions

To gain insight into the possible function of the *Notch* signaling pathway in the development of two polychaetes, *Capitella* sp. I and *P. dumerilii*, we began to analyse expression patterns of genes, which were proven to be part of this signaling pathway in other animal groups. For the future, functional studies should be the priority for the already isolated genes either by RNAi, morpholinos or even tranfection experiments. The DAPT experiments to knock out the *Notch* signaling pathway will be analyzed in detail with different antibody stainings not just for the nervous system, but also for muscles or even other protein specific antibodies available for *Platynereis dumerilii* like *nanos*. In situs with DAPT embryos are planned with e.g. *Pdu-Delta* and *hes*-genes. An analysis of the expression pattern of *Pdu-mef2* in DAPT embryos is of high interest to see how the expression of muscle specific genes are affected by the knock-down of *Notch*. One of the next step would also be to prove, directly or indirectly, the inhibtion of *Notch* in the cells. Since performing of DAPT experiments turned out to be so easy and reliable, it should also work and be done in *Capitella* sp. I embryos. It is also very important to perform in-situ hybridization with stages younger than 24h and juveniles in *P. dumerilii*. Double in-situ hybridization are also necessary to solve certain problems in the analysis of the gene expression patterns. RT-PCRs or Northern blots for all genes in different stages of *Capitella* sp. I and *P. dumerilii* will only determine the transcription level of *Notch* and its components.

To study the conservation of the *Notch* signaling itself, more genes should be isolated and examined representing components of the signaling in other phyla like *Numb*, *presenilin*, *mastermind* or *Serrate*. With these studies, conservation of certain genes can be examined or new functions of these components in other groups may also be identified.

5. Summary

The *Notch* signaling pathway is indispensable for the development of the Metazoa because of its involvement in various developmental processes including neurogenesis, myogenesis and segment formation. Expression patterns and its function have been studied in detail in several vertebrate and invertebrate model organisms. In this thesis, the receptor *Notch*, its ligand *Delta*, various downstream targets of hes genes and othercomponents involved were isolated from two polychaete species, *Capitella* sp. I and *P. dumerilii*. Expression patterns were analyzed and homologues of the same gene in both organisms were compared.

From *Capitella* sp. I, we isolated a 2.2 kb fragment of the 3` end of *CapI-Notch*, the complete ORFs of *CapI-Delta, CapI-hes1* and *CapI-fringe. Pdu-hes1, Pdu-hes2, Pdu-mef2* and *Pdu-Su(H)* were recovered by degenerate PCR in *P. dumerilii*. An initial fragment of *Pdu-Delta* isolated in the lab of Prof. Kress/ Berlin was RACEed out to obtain the complete ORF. The published sequence of *Pdu-Notch* in GeneBank was used to design specific primers, amplify a fragment and clone it into a vector for further experiments. We performed in-situ hybridizations on several consecutive larval stages of both polychaetes.

After analyzing all expression patterns, we predict the involvement of the *Notch* signaling pathway in chaetogenesis of *Capitella* sp. I and *P. dumerilii*. Both *Delta* homologues and *Notch* genes point to this conclusion as well as preliminary expression results of two *hes* genes in *Capitella* sp. I. Expression of genes studied in the central nervous system in consecutive stages account for the involvement of the *Notch* pathway in neurogenesis of both polychaetes as well. Most genes are expressed in the posterior growth zone of late larval stages of *Capitella* sp. I and *P. dumerilii* supporting the hypothesis of a possible function of *Notch* and its components in the later segmentation process of both annelids. Early expression patterns of all genes except *CapI-hes* do not account for a function of *Notch* signaling in segmentation in either polychaete. *CapI-hes1* is the only gene in our study which shows an iterated segmentally arranged expression pattern consistent with a possible involvement in the early segmentation process of *Capitell*a sp. I.

Judging from the expression patterns of *CapI-fringe, CapI-hes1, Pdu-Delta, Pdu-Su(H)* and *Pdu-hes2*, we can assume the existence of at least a second *Notch* gene in *Capitella* sp. I. It cannot be ruled out that still undiscovered orthologues of *Pdu-Delta* and *Pdu-Su(H)* in the genome of *P. dumerilii* exist.

6. Zusammenfassung

Anneliden, Arthropoden und Vertebraten stellen die drei großen segmentierten Gruppen im Tierreich dar. Ihr Segmentierungmodus basiert auf unterschiedliche Charakteristika. Arthropoden wie Cheliceraten, Myriapoden oder Crustaceen besitzen eine posteriore Wachstumszone, mit der sie Segmente bilden. Im Gegensatz dazu bildet aber der Embryo von *Drosophila* als Kurzkeim-Insekt alle Segmente in einem syncytialen Umfeld fast simultan aus. Kopfsegmente von *Schistocerca* als Langkeim-Insekt werden ähnlich gebildet im Gegensatz zum Rest des Körpers, der dann auch wieder die Wachstumszone benutzt, um neue Segmente zu bilden. Dies ist auch der Fall bei den meisten anderen Arthropoden. Segmentierung in Arthropoden scheint eher ektodermal abzulaufen.

Teloblastische Sprossung der Clitellaten (Annelida) stellt eine abgewandelte Form der Segmentbildung dar und wurde intensiv bei dem Egel *Helodella* untersucht. Eine basale Gruppe der Anneliden, die Polychaeten, scheint dagegen Segmente aus einer posterioren Proliferationszone (ohne Teloblasten) zu benutzen.

Die Segmente der Vertebraten werden Somiten genannt und sind mesodermalen Ursprungs. Sie werden in einer anterior zu posteriorer Richtung ausgebildet mit dem sogenannten posterioren somitischen Mesoderm als Ursprung. Diese Region im caudalen Teil des Embryos besteht aus undifferenzierten mesodermalen Zellen, die sich in anteriorer Richtung als Somiten abschnüren .

Es existieren drei Hypothesen über den Ursprung der Segmentierung. Die erste Theorie postuliert einen ancestralen segmentierten Urbilaterier dessen Mechanismen der Segmentierung man bei Anneliden, Arthropoden und Vertebraten wiederfindet. Dies bedeuttet aber auch den multiplen Verlust von Segmentierung in den heute unsegmentierten Tiergruppen. Die zweite Hypothese postuliert dagegen eine unabhängige Entwicklung des Segmentierungprozesses in Vertebraten und Protostomomiern. Letztere Gruppe mit Arthropoden und Anneliden haben in diesem Modell jedoch einen gemeinsamen Ursprung der Segmentierung. Die dritte Theorie besteht auf eine vollständig konvergente Entwicklung der Segmentierung.

In den letzten Jahren wurde versucht, mit molekularen Studien die genetische Kontrolleder Segmentierung zu entschlüsseln und durch Vergleich Argumente bzw. Plausibilitäten für

oder gegen diese Theorien zu gewinnen. Viele Gene und Signalwege, wie *engrailed, wnt* oder *hairy* wurden untersucht, die sowohl gegen als auch für eine der drei Hypothesen sprechen. Jüngere Studien des *Notch*-Signalweges in Spinnen, aber lässt die unwahrscheinliche Theorie der Homologie von Segmentierung in allen drei Gruppen wieder aufleben. Der *Notch*-Signalweg und alle damit verbundenen Faktoren sind eine zentrale Komponente der Somitogenese von Vertebraten. Dies wurde nun auch funktionell nachgewiesen in der basalen Arthropodengruppe der Cheliceraten. Ebenfalls gibt es Beweise über eine Konservierung des Signalweges und deren Komponenten *hairy, Delta, Supressor of hairless (Su(H))* und *Presenilin*.

Keine ausführlichen Studien des *Notch*-Signalweges und dessen Komponenten wurden bis jetzt in den Anneliden durchgeführt. Daher untersuchte ich diese Gene in den beiden anneliden Polychaeten *Capitella* sp. I und *Platynereis dumerilii*. Es wurden *Notch,- Delta,- hes,-* und *fringe*-Homologe aus *Capitella* sp. I isoliert wie auch *Delta, Notch, Su(H), hes* und *mef2*-Gene aus *P. dumerilii*. Alle Gene und deren Expressionsmuster wurden in unterschiedlichen Phasen der Embryonal- und Larvalentwicklung der beiden Polychaeten analysiert.

*CapI-Delt*a ist in frühen Stadien (St.4-5) in den Bauchplatten, im ZNS und lateral vom Mund exprimiert. Später verfeinert sich diese Expression zu einer notopodialen und einer heuropodialen Reihe jeweils lateral an beiden Seiten der Larve in der Position der späteren Borstensäcken. In älteren Larven ist das *CapI-Delta* Transkript im Mesoderm der posterioren Wachstumszone lokalisiert. Ein ähnliches Transkriptionsmuster kann bei *CapI-Notch* analysiert werden mit einer markanten Ausnahme: *CapI-Notch* ist stets breiter exprimiert als sein Ligand *CapI-Delta*, aber dennoch in denselben Regionen vom ZNS, lateral vom Mund, in zwei Reihen der zukünftigen Borstensäcke und später im Mesoderm der posterioren Wachstumszone. Dieses Überlappen der Expressionsmuster bestätigt eine mögliche Ligand-Rezeptor Beziehung für *CapI-Delta* und *CapI-Notch*.

Pdu-Notch ist sehr früh und sehr stark in fast allen Regionen des Embryos und auch später noch im Mundbereich, dem ZNS und den Borstensäcken exprimiert. Dagegen ist *Pdu-Delta* ab 36 Stunden nur noch in den Borstensäcken exprimiert. Es wurde jedoch keine Expression im ZNS detektiert. Beide Gene sind bei 72h alten Larven ebenfalls in der posterioren Wachstumszone exprimiert. Somit konnte die Expression von *Delta* und *Notch* Homologen der beiden Polychaeten in den zukünftigen Borstensäcken bestätigt werden und eine mögliche Funktion des Notch-Signalweges in der Chaetogenese von *Capitella* sp.

I and *P. dumerilii* ist dadurch sehr wahrscheinlich. *Pdu-Notch* und *Pdu-Delta* sind nach der Bildung der Borsten immer noch exprimiert im Gegensatz zu *CapI-Delta* und *CapI-Notch*, deren Expression kurz vor Borstenbildung abschwächt und dann auch verschwindet. Dies könnte etwas mit der schnelleren Entwicklung von *P. dumerilii* im Vergleich zu *Capitella* sp. I zu tun haben.

Ein *fringe* Homolog wurde nur aus *Capitella* sp. I isoliert. Die Expression von *fringe* überlappt in fast allen Regionen mit *CapI-Notch* und *CapI-Delta*. Somit ist es sehr gut möglich, dass *fringe* vergleichbar zu Vertebraten auch einen regulativen Einfluss auf den *Notch*-Signalweg hat. Die Expression im Bauchmark des ZNS spricht für ein zweites *Notch*-Gen in *Capitella* sp. I. Eine Funktion für *fringe* außerhalb des *Notch*-Weges wurde noch nicht entdeckt und ist somit unwahrscheinlich.

Verschiedene *hes*-Gene wurden aus beiden Organismen isoliert. *CapI-hes1* und *Pdu-hes2* wurden durch phylogenetische Analysen basal von *hairy*-Genen und Invertebraten-*hes*-Homologen eingeordnet. *Pdu-hes1* konnte dagegen direkt als Invertebraten *hes*-Gen klassifiziert werden. Vorläufige Expressionsdaten von *CapI hes2* zeigt ebenfalls ein Muster in den zukünftigen Borstensäcken als auch später in der posterioren Wachstumszone (E.C. Seaver, unpubl.). Diese Ergebnisse könnten ein Hinweis auf mögliche downstream targets von *Notch* sein, wie das auch bei Vertebraten oder auch Arthropoden der Fall ist.

Pdu-hes1 und *Pdu-hes2* dagegen zeigen fast keine überlappenden Expressionsdomänen mit *Pdu-Notch* oder *Pdu-Delta*. *Pdu-hes2* ist hauptsächlich im Mundbereich und der ventralen Mittellinie exprimiert und eventuell gesteuert durch ein zweites *Notch*-Gen in *P. dumerilii* oder durch einen vollkommen *Notch*-unabhängigen Signaltransduktionsweg. *Pdu-hes1* ist im Mesoderm vor allem um die Borstensäcke und später aber auch in der posterioren Wachstumszone exprimiert. Diese Expressionsdomäne wiederum überlappt mit *Pdu-Notch* und *Pdu-Delta*, was eine spätere Kontrolle von *Pdu-hes1* durch *Pdu-Notch* nicht ausschließt.

CapI-hes1 ist das einzige Gen von allen untersuchten Homologen, das ein segmental angeordnetes Expressionsmuster zeigt. Die Expression ist lokalisiert in Streifen in den zukünftigen Segmenten der Larve. Es folgt in seiner Expression dem Muster der postulierten Segmentierung von *Capitella* sp. I. Für *CapI-hes1*bedeutet dies eine gestreifte Expression in den Bauchplatten für die Bildung der ersten neun bis zehn Segmente und

später ist das Cap-hes1 Transkript lokalisiert in der posterioren Wachstumszone. Von der terminalen Wachstumszone aus werden später sowohl die letzten drei bis vier larvalen und alle postlarvalen Segmente gebildet.

Das isolierte *Pdu-Su(H)*-Gen ist transient im ZNS, Mund und im Enddarm exprimiert. Ab 60h in der Entwicklung kann das Transkript von *Pdu-Su(H)* in zwei bilateralen Regionen im Kopf und vermutlich ZNS detektiert werden. Eine Lokalisation in der posterioren Wachstumszone vergleichbar zu Vertebraten oder Cheliceraten konnte jedoch nicht bestätigt werden. Ein zweites *Su(H)*-Gen als CSL Regulator für *Notch* ist sehr wahrscheinlich.

Pdu-mef2 konnte in den entstehenden Muskeln überlappend exprimiert mit *Pdu-hes1* vor. Die gemeinsame Lokalisation von einem downstream target von *Notch* und *Pdu-mef2* gibt Hinweis auf die Regulierung von *Pdu-mef2* durch *Pdu-Notch* oder ein zweites *Notch* Homolog in *P. dumerilii*.

Vorläufige Ergebnisse mit DAPT, einem NICD (*Notch* intracellular domain) Inhibitor, in *P. dumerilii* zeigen wesentliche Defekte in der Borstenbildung, Neurogenese, Myogenese und Kieferausbildung. Expressionsmuster von *Pdu-Delta, Pdu-Notch* und *Pdu-hes1* als auch *Pdu-mef2* unterstützen diesen Phänotypen.

Die frühe Expression von *CapI-Delta, CapI-Notch, CapI-fringe, Pdu-Notch* und *Pdu-hes1* kann einen Einfluss des *Notch*-Signalweges in der frühen larvalen Segmentierung von beiden Polychaeten nicht ausschließen, aber die Expressionsmuster machen es eher unwahrscheinlich. Die spätere Expression von den meisten Genen in der posterioren Wachstumszone dagegen in *Capitella* sp. I und *P. dumerilii* macht eine wesentliche Beteiligung des *Notch*-Weges und seinen Komponenten während der postlarvalen Segmentierung in Polychaeten sehr wahrscheinlich. Ergebnisse von in-situs mit juvenilen Würmern von *Capitella* sp. I zeigen ebenfalls Expression in der posterioren Wachstumszone und den neu gebildeten Segmenten.

Mit dieser Arbeit konnte ich zum ersten Mal eine Konservierung des *Notch*-Signalweges und seiner Komponenten in der Entwicklung basaler Vertreter der Lophotrochozoa nachweisen. Expressionsmuster deuten auf eine Ligand-Rezeptor Beziehung von *Notch* und *Delta* in *Capitella* sp. I und *P. dumerilii* hin. Die vergleichenden Analyse der Gene

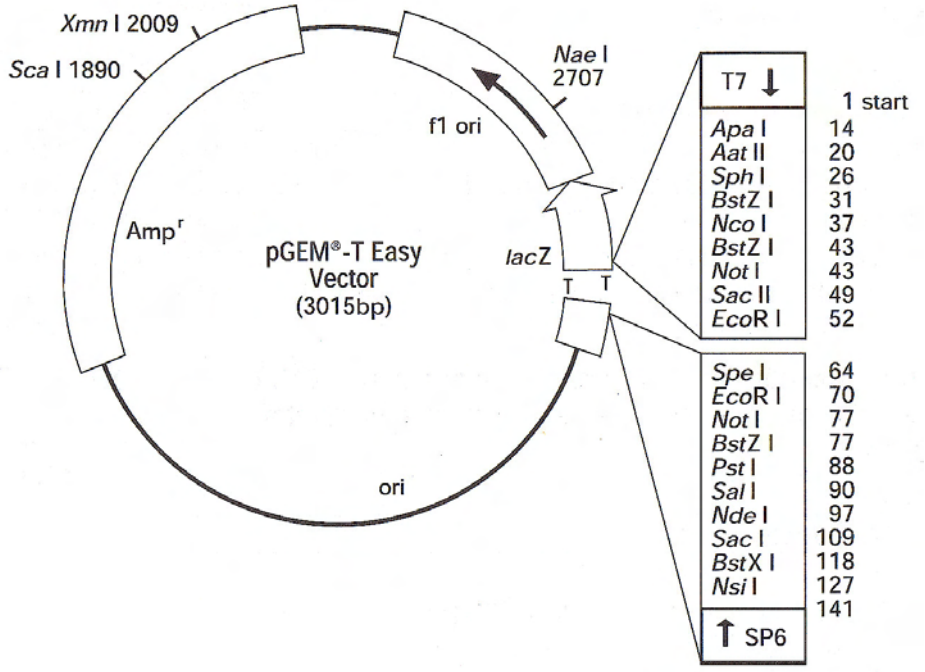
und deren Expressionmuster deutet auf eine Rolle bei der Neurogenese, Myogenese und Chaetogenese in beiden Polychaeten hin.

Somit ist ein Einfluss von *Notch* und seinen Komponenten als auch die Verbindung zu anderen Signalwegen in Anneliden sehr wahrscheinlich wie auch eine Funktion in Segmentierung, Neurogenese oder Sinnesorganbildung. Der *Notch*-Signalweg ist daher vermutlich auch involviert in mehreren entwicklungsbiologisch relevanten Mechanismen bei Lophotrochozoen.

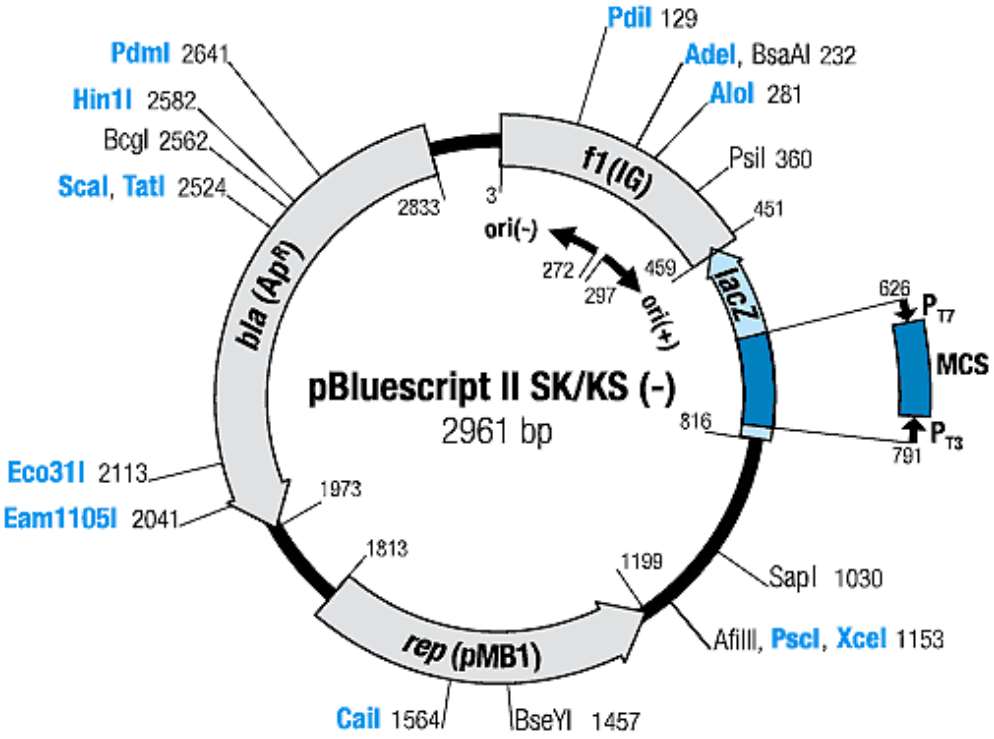
7. Supplemental material

7.1 Vector maps

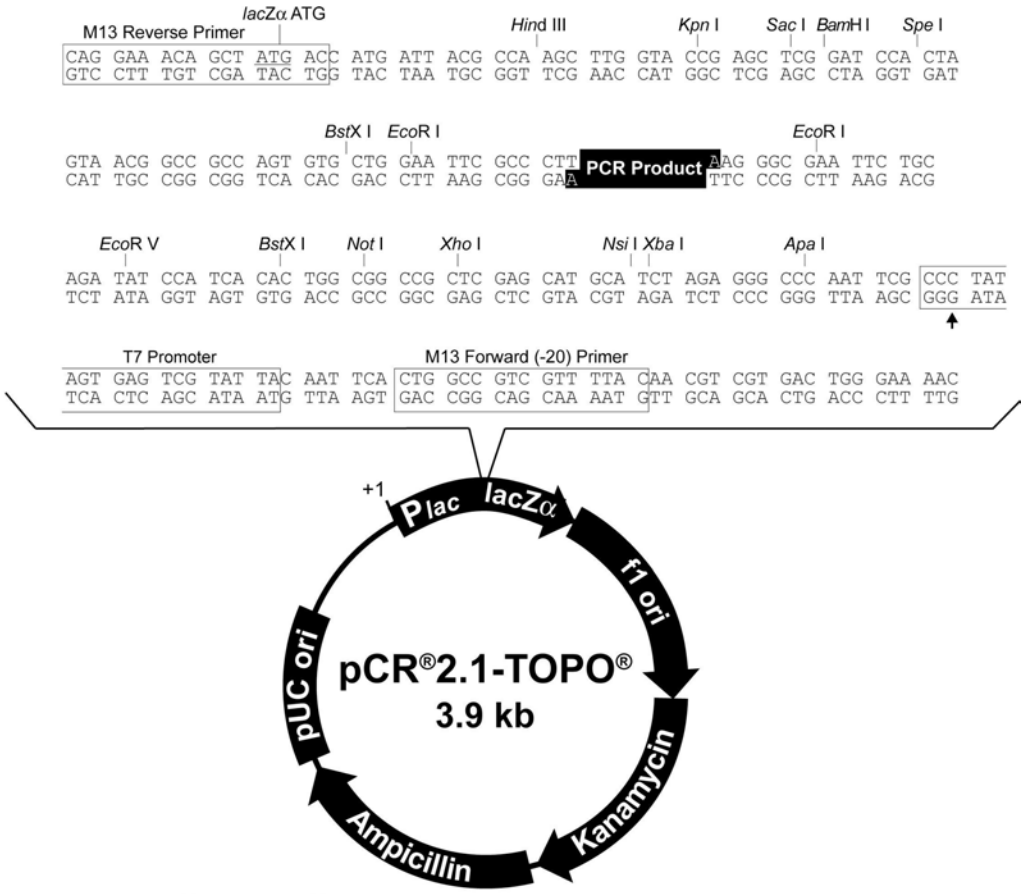
a) pGEM®-T Easy vector (Promega)



b) pBluescript®SK(-) vector (Stratagen)



c) pCR®2.1-TOPO (invitrogen)



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

7.2 Genebank Accession numbers

***Capitella* sp. I:** *CapI-Notch* - DQ3846218, *CapI-hes1* - DQ384620, *CapI-Delta* - DQ384619

Platynereis dumerilii: *Pdu-Notch* - CAJ38792

Delta: Dm-Delta – X06289, Gm-Delta – AJ536341, Cs-Delta1 – AJ50729, Cs-Delta2 – AJ5072902, Xl-Delta1- C42229, Mm-Delta1 – NM007865

Hes: Dmhairy – AY119633, Dmdeadpan – AY071330, Xlhairy1 – XLU36194, Xlhairy2a – AF383159, Xlhairy2b – AF383160, Tchairy – AJ457831, Cshairy – AJ252154, Hshes4 – NP066993, Gghairy1b – AY225440, BfhairyA – AY349467, DmEsplm5 – X16552, DmEsplm8 – X16550, XlHESR1a – BA1378540, Drher1 – NM131078, Drher5 – NM131077, Drher6 – NM131079, Drher7 – NM131609, Drher9 – NM131873, Dmhesr1 – AF151523, Mmhes1 – NM008235, Mmhes3 – NM008237, Mmhes5 – NM010419, Mmhes7 – NM033041, ESR1 – AF383157, ESR2 – AF383158, ESR5 – AF137072, RnSharp1 – AF009329, HsStra13 – NM144998, MmStra13 – AF010305, Mmhey1 – AJ271867, Drgridlock – AF237948, Xltwist – M27730, Nvtwist – AY465180, Dmtwist – X14569, Hstwist – X91662, Hrotwist – AF410867

Fringe: Dmfringe – AAA64525, Bffringe –CAD97418, Drlfringe –NP571046, Rnlfringe –BAB63256, Rnmfringe –AAH61801,Rnrfringe –NP06821,

Su(H): DmSu(H) – AAD39717, CsSu(H)1 –CAG30665, CsSu(H)2 –CAG30667, CiSu(H) –AAC34125, XlSu(H)1 - AAB05478, DrSu(H) –AAM97536, MmSu(H) –P31266,

Mef2: Drmef2A – AAC05225, Hsmef2A – CAA48517, Bmmef2 – NP001036905, Atmef2 – AB125745, Pcmef2 – AJ428495, Nvmef2 – AAR24454, Mmmef2D – NP932111

7.3 Sequences

CapI-fringe:

TCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGG
CAAGATGTTTACATCAGCGTTAAAACTACCAGGAAATATCACCGAGATCGCCTTGACCTGCTACTCAAGACAT
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TACGATTCGTTTATTGCCTCAGAGAAAAGGTGGTTTTGCCACGTTGATGACGATACATATGTGAATACGGGAG
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GCTGCAGATGGGCGACAAGGAGAATCCTGGGCAAAAGATTGCGTTTTGGTTCGCCACAGGAGGCGCTGGCTTC
TGTATTAGTCGTGGTCTGGCTCTGAAAATGATGCCACACACCAGTGGAGGAAGGTTGAAAACCGTCTGTGAGC
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ACATATTTCAAGAATAGAAAATGTTCTTTGCTCGATATTCTCTTCTTCTTTTGTTCATTTTTCGTTCNCAAAA
TTACTTACATGTTCNCAAAATGNGCCTTTTTTTTGCCAAATAGTTAATAAAGNGGAAAAAAAAAAAAAAAAAA
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Pdu-Delta:

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Pdu-hes1:

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Pdu-Su(H):

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Pdu-mef2:

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9. Abbreviations

- amp — ampicillin
- AP-buffer — alkaline phosphatase-buffer
- BCIP — 5-bromo-4-chloro-3-indolyl phosphate
- BCP — 1-brom-3-chlorpropan
- bp — base pair
- BSA — bovine serum albumin
- CNS — central nervous system
- CTAB — cetyl trimethyl ammonium bromide
- DEPC — diethylpyrocarbonate
- DIG — digoxygenine
- DMF — dimethylformamide
- DMSO — dimethyl Sulfoxide
- DNA — desoxyribonucleic acid
- DNase — desoxyribonuclease
- dNTP — deoxyribonucleotide triphosphate
- EDTA — ethylenediamine tetraacetic acid
- EtOH — ethanol
- FSW — filtered sea water
- µg, mg, g — microgram, milligram, gram
- hr, hrs — hour, hours
- hybe — hybridization
- IPTG — isopropyl-β-D-thiogalactoside
- kb — kilobase
- µl, ml, l — microliter, milliliter, liter
- LB — luria bertani
- MCS — multiple cloning side
- MeOH — methanol
- min — minute
- µM, mM, M — micromolar, millimolar, molar
- MOPS — 3-(N-Morpholino)propanesulfonic acid
- NBT — 4-Nitro blue tetrazolium chloride
- NSW — natural sea water
- o/n — overnight
- ORF — open reading frame
- PFA — paraformaldehyde
- PBS — phosphate buffered saline

- PBT — PT plus BSA
- PCR — polymerase chain reaction
- PT — PBS plus Triton X-100
- PTw — PBS plus Tween-20
- PVP — polyvinyl pyrrolidone
- RNA — ribonucleic acid
- RNase — ribonuclease
- rpm — revolutions per minute
- RT — room temperature
- SDS — sodium dodecyl sulfate
- sec — second
- SOB — super optimal broth
- SOC — super optimal broth plus glucose
- SSC — sodium chloride sodium citrate
- SSCT — sodium chloride sodium citrate plus Tween
- TAE — tris-acetate-EDTA
- TE — tris-EDTA
- TEA — triethanolamine
- V — volt
- X-Gal — 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Eidesstattliche Erklärung

Hiermit erkläre ich, Katrin Thamm, an Eides statt, dass ich die vorliegende Arbeit zur Erlangung des naturwissenschaftlichen Doktorgrades selbständig verfasst und keine anderen Hilfsmittel als die angegebenen Quellen verwendet habe.

Katrin Thamm

Gießen im Juni 2007

Curriculum vitae

NAME:	Katrin Thamm
GEBURTSDATUM UND-ORT:	06.04.1978 in Hildburghausen
STAATSANGEHÖRIGKEIT:	deutsch
ADRESSE:	Institut für Allgemeine und Spezielle Zoologie/ Abteilung Entwicklungsbiologie Stephanstraße 24 35390 Gießen

AUSBILDUNG:

ZEITRAUM	INSTITUTION	ABSCHLUß
1984-1990	Geschwister-Scholl-Oberschule Zschopau	
1990-1996	Gymnasium Zschopau	Abitur
04.1996 - 01.2001	Friedrich-Schiller-Universität Jena	Studium der Biologie
02.2001 - 04.2002	FSU-Jena/ Christian-Albrechts-Universität Kiel	Diplom, Prüfungsfächer: Zoologie, Genetik, Mikrobiologie und Anthropologie
07.2002 - 01.2003	University of California in Los Angeles	Forschungsaufenthalt im Labor von Prof D. Jacobs
02.2003 – 02.2005	University of Hawai'i	Forschungsaufenthalt im Labor von Prof. Elaine Seaver incl. Beginn der Doktorarbeit ab 09.2003
03.2005 - gegenwärtig	Justus-Liebig-Universität Gießen	Promotion (vorraussichtlich 07.2007), Prüfungsfächer: Zoologie, Entwicklungs-biologie und Molekular-biologie