Neurogenesis in the millipede *Glomeris marginata* (Myriapoda: Diplopoda)

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Hilary Livia Dove

aus Wien, Österreich

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1. Berichterstatter: Prof. Dr. D. Tautz.
2. Berichterstatter: Prof. Dr. S. Roth

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Zusammenfassung

Ziel der vorliegenden Arbeit war es, die frühen Abläufe der Neurogenese an einem Vertreter der Myriapoden auf morphologischer und molekularer Ebene zu untersuchen. Unter den Myriapoden existiert noch kein gut etablierter Modellorganismus. Daher musste noch vor Beginn der eigentlichen Untersuchungen eine geeignete Art augewählt werden und Basisprotokolle für die verschiedenen histologischen und molekularen Studien etabliert werden. Als geeigneter Repräsentant der Myriapoden fiel die Wahl auf den Tausendfüssler *Glomeris marginata*, unter anderem weil adulte Tiere in der näheren Umgebung gesammelt werden konnten. Einen weiteren Vorzug bot die Tatsache, dass zu dieser Art bereits eine umfangreiche Beschreibung der allgemeinen Entwicklungsabläufe vorlag.

Begonnen wurde mit den Untersuchungen zur Morphologie des ventralen Neuroektoderms bei *G. marginata*. Embryonen unterschiedlicher Stadien wurden mit Hilfe verschiedener Marker und histologischer Methoden gefärbt und entsprechend ausgewertet. Hierbei konnte ich feststellen, dass - ähnlich wie im Neuroektoderm der Spinne - Gruppen neuraler Vorläuferzellen von der apikalen Oberfläche des ventralen Neuroektoderms invaginieren. Solche Zellgruppen entstehen in vier zeitlich aufeinander folgenden Wellen an stereotypen Positionen innerhalb des jeweiligen Segmentes. Offenbar nimmt die gesamte neurogene Region ein neurales Schicksal an, im Gegensatz zu Beobachtungen bei Insekten, wo letztendlich nur ausgewählte Zellen des Neuroektoderms den neuralen Entwicklungsweg einschlagen. Im Unterschied zu der Spinne sind bei *G. marginata* mitotisch aktive Zellen mit invaginierenden Zellgruppen assoziert, was die Frage aufwirft, ob in Myriapoden neurale Stammzellen existieren.

Der molekulare Ansatz meiner Studie befasste sich damit, aus *G. marginata* Homologe von Genen zu isolieren, die bei anderen Arthropoden eine Rolle in der Neurogenese spielen. Diese Gene liessen sich in vier Gruppen gliedern: proneurale Gene, die in der frühen Neurogenese eine Rolle spielen, neurogene Gene, Gene für die dorso-ventrale Musterbildung und spätere Marker der Zellschicksalspezifizierung. Die Expressionsmuster dieser Gene wurden mit denen von Homologen anderer Arthropoden verglichen. Dieser Vergleich deutet an, dass es ein konserviertes genetisches Netzwerk für die Entwicklung des Nervensystems in Arthropoda gibt.

Abstract

The development of the nervous system, termed neurogenesis, has been studied in detail on a morphological level in insects, crustaceans and recently also in chelicerates, however, comparable data is missing from myriapods. In addition, nothing is known about the molecules involved in neurogenesis in this group. One reason for this lack of information is that there is no established model system for the study of myriapod development. Thus, before investigating myriapod neurogenesis, I had to decide on a model organism and to establish numerous basic protocols for the intended studies. I chose the millipede *Glomeris marginata* because adults and eggs are locally available and the embryonic development of this species has been described in detail.

After this, I analysed the morphology of the ventral neuroectoderm of *G. marginata* embryos stained with various markers on whole mounts and serial sections. I found that groups of neural precursors invaginate from the apical surface of the ventral neuroectoderm in a stereotypic pattern, which is comparable to what I observed in a distantly related diplopod, *Archispirostreptus sp*. Neural precursors arise sequentially in four waves, however, ultimately, all cells of the neurogenic region enter the neural pathway. Thus the mode of neurogenesis found in millipedes is similar to what has been reported for spiders. The major difference is that in *G. marginata*, cell divisions are associated with the invaginating cell groups, implying that stem cells are present in the ventral neuroectoderm.

The molecular part of my research involved the isolation and the analysis of *G. marginata* homologues of genes that are necessary for neurogenesis in other arthropods. Genes isolated included proneural genes involved in early neurogenesis, neurogenic genes, dorso-ventral patterning genes and markers of later cell fate specification. The expression patterns of these genes was compared to that of homologues in other arthropod species, and, if available, to data from the spider *Cupiennius salei*. I found that genes are expressed in the expected domains, implying that there is a conserved genetic network controlling the development of the nervous system in arthropods.

1. Introduction

1.1. Phylogenetic position of Myriapoda

The phylum Arthropoda is one of the largest animal clades, which comprises four major groups: the insects, the crustaceans, the chelicerates and the myriapods. While the monophyly of Arthropoda is generally accepted, the relationships between the individual groups are not clear (Nielsen, 2001). Recent molecular and morphological data have challenged the traditional view that insects and myriapods are sister groups (Akam *et al.*, 1988; Dohle and Scholtz, 1988; Patel *et al.*, 1989a; Patel *et al.*, 1989b; Scholtz, 1990; Whitington *et al.*, 1991; Scholtz, 1992; Friedrich and Tautz, 1995; Boore *et al.*, 1995; Damen and Tautz, 1998; Damen *et al.*, 1998; Telford and Thomas, 1998; Abhzanov *et al.*, 1999; Abhzanov and Kaufman, 1999; Abhzanov and Kaufman, 2000; Damen *et al.*, 2000; Hwang *et al.*, 2001; Harzsch, 2002). This view was based on supposedly shared characters such as loss of second antennae, formation of Malpighian tubules, postantennal organs, and tracheae.

Re-evaluation of these characters, however, shows that they may be prone to convergence (Friedrich and Tautz, 1995; Dohle, 2001). Instead, it is possible to find synapomorphies between insects and higher crustaceans that are absent in equivalent form in myriapods (Dohle, 2001). The synapomorphies of insects and crustaceans are the pattern of axonogenesis in early differentiating neurons, the fine structure of ommatidia, the expression patterns of segmentation genes and possibly the presence of neuroblasts (Dohle, 1997; Dohle, 2001). Some molecular data sets even suggest that the chelicerates and the myriapods are sister groups (Friedrich and Tautz, 1995; Cook *et al.*, 2001; Hwang *et al.*, 2001). However, morphological data supporting this hypothesis are still missing.

The monophyly of myriapods is still being debated. Myriapoda is divided into four undisputed monophyletic groups: Pauropoda, Symphyla, Diplopoda and Chilopoda (Dohle, 1997). Traditionally, Myriapoda has been regarded as a monophyletic group, however, this has long been questioned (Pocock, 1893; Snodgrass, 1952; Dohle, 1980; Shear, 1997). Comparison of morphological data led Dohle to the conclusion that there is no evidence for the monophyly of Myriapoda. Rather, Diplopoda, Symphyla and Pauropoda can be grouped together as Progoneata, while Chilopoda form an independent group (Dohle, 1997; Dohle 2001). Analyses based on molecular data, on

the other hand, have generally provided evidence for the monophyly of Myriapoda (Regier and Schultz, 1997; Giribet and Ribera, 2000; Regier and Schultz, 2001).

In this report, Myriapoda will be considered a valid taxon, however, and the mode of neurogenesis found in *Glomeris marginata* will be compared to what is known from the spider *Cupiennius salei* and other arthropods, in an attempt to help resolve the evolutionary relationships between myriapods, chelicerates and insects.

1.2. The arthropod nervous system

1.2.1. Comparison of neurogenesis in arthropods

In arthropods, the central nervous system (CNS) consists of the brain and the ventral nerve cord, which develops from the neurogenic region, called the ventral neuroectoderm (VNE). Each segment is divided into two bilaterally symmetrical regions, the so-called hemisegments, by the ventral midline. Neurons are generated by the proliferation of progenitor cells in each hemisegment (Anderson, 1973). Much of our knowledge of arthropod neurogenesis comes from insects and comparable data from other taxa, especially molecular analyses, is often missing. Neural development in *Drosophila melanogaster* is controlled by many genes, and, aside from the recent data from the spider *Cupiennius salei*, little is known about the molecular mechanisms required for neurogenesis in other arthropods. For this reason I will first give a brief description of neurogenesis in each arthropod group and then discuss the molecular mechanisms involved in nervous system development in greater detail.

1.2.2. Insect neurogenesis

Insect neurogenesis has been best studied in the fruitfly *D. melanogaster* and the locust *Schistocerca gregaria,* and further analyses indicate that the nervous systems of all insects may develop in a similar manner, with minor variations (Thomas *et al.*, 1984; Truman and Ball, 1998). In the insect VNE, proneural genes confer neural competence to groups of cells, the so-called proneural clusters. This marks the beginning of neurogenesis. One cell from each proneural cluster inhibits the other members of the cluster from adopting a neural fate via lateral inhibition and becomes the neural stem

cell, the so-called neuroblast (Hartenstein and Campos-Ortega, 1984; Goodman and Doe, 1985a; Goodman and Doe, 1993; Campos-Ortega, 1993).

Five distinct waves of neuroblast formation lead to the delamination of approximately 30 neuroblasts in a stereotypic pattern of six to seven rows of four to five cells each from the apical surface of a single hemisegment (Hartenstein and Campos-Ortega, 1984; Goodman and Doe, 1985a; Goodman and Doe, 1993; Campos-Ortega, 1993). The first neuroblasts are formed in three columns; however, this pattern is obscured in later waves of formation (Goodman and Doe, 1985a; Goodman and Doe, 1993; Campos-Ortega, 1993). Each neuroblast has an individual identity based on its location in the hemisegment, and can be recognized individually both by its location and by the subset of neural markers it expresses (Goodman and Doe, 1985b; Goodman and Doe, 1993).

After delamination, neuroblasts come to lie at basal positions where they divide asymmetrically, generating two unequal sibling cells (reviewed in Campos-Ortega, 1993; Goodman and Doe, 1993). One of these cells retains its stem-cell like features, and the other, the ganglion mother cell (GMC), divides once to produce two terminally differentiated neurons or glial cells (reviewed in Goodman and Doe, 1993; Campos-Ortega, 1993). Each neuroblast divides a specific number of times to produce a defined number of neurons (reviewed in Campos-Ortega, 1993; Goodman and Doe, 1993). The earliest axonal pathways are established by a set of neurons that arise in a stereotypic pattern in each hemisegment. Subsets of these pioneer neurons can be recognized by their axonal projections and the expression of specific markers (Thomas *et al.*, 1984; Whitington, 1995; Whitington, 1996).

The *D. melanogaster* peripheral nervous system (PNS) consists of four major types of sensory elements: external sensory organs, chordotonal organs (stretch receptors), multiple dendritic neurons and photoreceptors. These elements arise from clusters of cells, which, similar to the proneural clusters in the CNS, attain neural competence through the action of proneural genes. As in the CNS, lateral inhibition leads to the singling out of one sensory organ precursor cell, which then divides in a stereotyped fashion to produce a fixed number of progeny cells (Jan and Jan, 1994 and references therein).

1.2.3. Crustacean neurogenesis

Neuroblasts have been described for several malacostracan crustaceans, but their existence in Entomostraca is still disputed (Gerberding, 1997; reviewed in Harzsch, 2003b). For the purposes of this description, crustaceans will be treated as a monophyletic group; however, it should be mentioned that this is not yet resolved and that the mode of neurogenesis presented here may only apply to a subset of crustaceans (Regier and Schultz, 1997; reviewed in Harzsch, 2003b). Crustaceans, like insects, have neuroblasts that are recognizable at the apical surface of the VNE by their enlarged size (Scholz, 1992; Harzsch and Dawirs, 1994; reviewed in Harzsch, 2003b).

Similar to insects, approximately 25 - 30 neuroblasts are found in a stereotypic pattern of four to five irregular rows of six to seven cells per hemisegment (Scholz, 1992; Harzsch and Dawirs, 1994; reviewed in Harzsch, 2003b). Unlike insect neuroblasts, crustacean neural stem cells are generated by specialized precursor cells, the ectoteleoblasts, via a defined lineage and do not delaminate from the apical surface (Dohle and Scholtz, 1988; Scholz, 1992; Harzsch and Dawirs, 1994; Gerberding, 1997; Duman-Scheel and Patel, 1999; Harzsch, 2003b). At least in some species, neuroblasts divide asymmetrically to generate ganglion mother cells towards the interior of the embyo (Scholz, 1990; Scholz, 1992; Scholz and Dohle, 1996).

In addition, while insect neuroblasts only give rise to neural progeny, at least some lineages of their crustacean counterparts can give rise to both neuronal and epidermal cells (reviewed in Harzsch, 2003b). The lineage of crustacean neuroblasts is unclear, with the exception of the median neuroblast (Geberding and Scholtz, 2001). However, a number of neurons have been identified in several species that are homologous to those found in insects based on their location, axonal outgrowths and the expression of neuronal markers (Whitington *et al.*, 1993; Whitington, 1995; Duman-Scheel and Patel, 1999; Gerberding and Scholtz, 2001; Harzsch, 2003a).

1.2.4. Neurogenesis in Chelicerata

It has been shown recently that neurogenesis in the spider *Cupiennius salei* (chelicerate) shares several features with insects and crustaceans, but that there are also important differences. Similar to the generation of neuroblasts in insects, approximately 30 groups of cells invaginate from the apical surface of the VNE, the so-called invagination sites. These invagination sites arise sequentially in four waves at stereotyped positions in

regions of the VNE that are prefigured by proneural genes (Stollewerk *et al.*, 2001). Neurogenic genes restrict the proportion of cells that adopt the neural fate at each wave of neural precursor formation; however, in contrast to insects, groups of cells, rather than single cells, adopt the neural fate at a given time (Stollewerk *et al.*, 2001; Stollewerk, 2002).

In addition, neural stem cells, comparable to *D. melanogaster* neuroblasts, could not be detected in the VNE of the spider. Cells divide at the apical surface and are recruited to the neural fate at a given time; however, there is no decision between epidermal and neural fate in the VNE of the spider as in *D. melanogaster*. Instead, all cells of the neurogenic region enter the neural pathway (Stollewerk, 2002). Invaginating cell groups express neural markers, and no asymmetric division of neural precursor cells or asymmetric segregation of proteins could be observed (Weller, 2002).

1.2.5. Myriapod neurogenesis

In all four myriapod groups (Diplopoda, Chilopoda, Symphyla, and Pauropoda), the general development of the VNE follows the same pattern. Ventral to the limb buds, thickenings form as a result of cell proliferation. When the embryo begins to bend about a transverse fold in the middle of the trunk, these thickenings flatten (Anderson, 1973). After completion of ventral flexure the middle part of the hemisegment sinks into the embryo forming a groove (Dohle, 1964). Cell proliferation takes place within this groove pushing newly formed cells towards the basal side and leading to the formation of stacks of cells that project out as rays from the edges of the groove. This structure is called the “ventral organ”. During the course of neurogenesis the ventral organs are gradually incorporated into the embryo while epidermal cells overgrow the ventral nerve cord (Dohle, 1964).

Neurogenesis has been analysed in a variety of representatives of all myriapod groups, but failed to reveal stem cell-like neural precursors with morphological characteristics of insect or crustacean neuroblasts (Heymons, 1901; Tiegs, 1940; Tiegs, 1947; Dohle, 1964; Whitington *et al.*, 1991). Furthermore, Whitington and co-workers showed that in the centipede *Ethmostigmus rubripes* the earliest central axon pathways do not arise from segmentally repeated neurons as in insects but by the posterior growth of axons originating from neurons located in the brain (Whitington *et al.*, 1991). In addition, the axonal projections and the cell body positions of the segmental neurons

clearly diverge from the pattern described in insects and crustaceans (Whitington *et al.*, 1991; Whitington, 1995).

1.2.6. Genes involved in arthropod neurogenesis

In *D. melanogaster*, early neurogenesis is controlled by several bHLH (basic Helix Loop Helix) genes that encode transcription factors with a basic domain necessary for DNA binding and two helices that allow for the formation of heterodimers with other bHLH proteins. In the VNE of *D. melanogaster*, members of the *achaete-scute* complex (AS-C; *achaete, scute and lethal of scute*) are expressed in a stereotyped pattern of proneural clusters and are necessary for neural precursor formation in the CNS. In loss-of-function mutants fewer neural precursors are formed (Cabrera *et al.*, 1987; Alonso and Cabrera, 1988; Jimenez and Campos-Ortega, 1990; Skeath and Carroll, 1992; Campos-Ortega, 1993; Skeath and Doe, 1996).

Achaete-Scute Homologs (ASH) were also identified in the spider *Cupiennius salei*, which share sequence similarities with proneural genes from other species. Both *CsASH1* and *CsASH2* are expressed in invaginating cell groups. While functional analyses showed that *CsASH1* is required for the formation of invagination sites in the VNE, *CsASH2* plays a later role in the differentiation of the neural precursors (Stollewerk *et al.*, 2001; Stollewerk *et al.*, 2003).

Another class of genes that is necessary for neuroblast formation in the *D. melanogaster* CNS and that acts in parallel to the genes of the *AS-C* are members of the group B of the *Sox* family of transcription factors. Two members of this group from *D. melanogaster, Dichaete* and *Sox Neuro*, were found to have overlapping and partially redundant functions in specification and patterning of the VNE. This is reflected by their expression domains; *Dichaete* in the ventral midline and medial part of the VNE and *Sox Neuro* in the complete VNE. *Dichaete* and *Sox Neuro* double mutants show severe neural hypoplasia throughout the CNS as well as loss of proneural clusters and medially derived neuroblasts (Soriano and Russell, 1998; Cremazy *et al.*, 2000; Buescher *et al.*, 2002; Overton *et al.*, 2002).

In the *D. melanogaster* PNS, *AS-C* genes are necessary to promote external sensory organ precursor formation (Rodriguez *et al.*, 1990; Brand *et al.*, 1993). *Pannier*, the only GATA transcription factor found in insects, activates the *AS-C* genes in the PNS in a complex spatial pattern allowing for the species-specific positioning of sensory bristles (Ramain *et al.*, 1993; Gomez-Skarmeta *et al.*, 1995; Heitzler *et al.,*

1996; Garcia-Garcia *et al*., 1999; Wülbeck and Simpson, 2002). In the absence of *pannier*, no sensory bristles are formed. In addition, *pannier* is expressed in a longitudinal dorsal domain in several species of flies and in the mosquito *Anopheles gambiae.*

It has been suggested that *pannier* has an ancient role as a selector gene for dorsal body pattern in insects because it is essential for the formation of dorsal tissues and dorsal closure in *D. melanogaster. Pannier* mutants do not survive because they have severe defects in cuticle formation. (Ramain *et al.*, 1993; Winick *et al.*, 1993; Heitzler *et al.,* 1996; Calleja *et al.*, 2000; Wülbeck and Simpson, 2002). After dorsal closure, *pannier* is expressed in broad domains on the notum encompassing the more restricted areas of *AS-C* expression, where sensory bristles will form. Overexpression of *pannier* at this stage of development leads to the formation of ectopic bristles due to an overexpression of *AS-C* genes (Heitzler *et al.*, 1996; Garcia-Garcia *et al*., 1999; Simpson *et al.*, 1999; Wülbeck and Simpson, 2002).

Atonal-type bHLH proteins, encompassing the genes *atonal* and *amos,* are also expressed in the *D. melanogaster* PNS. *Atonal* is required for the formation of internal sensory organ precursors and is expressed in the precursors of the chordotonal organs and photoreceptors (Jarman *et al.*, 1995). *Amos* is necessary for the formation of the multiple dendritic neurons (Huang *et al.*, 2000). Null mutations of the *AS-C* genes and *atonal*-type bHLH genes show the same phenotype as mutations in the bHLH gene *daughterless*, which remove the complete PNS (Caudy *et al.*, 1988a; Caudy *et al.*, 1988b; Vaessin *et al.*, 1994; Jarman *et al.*, 1995; Huang *et al.*, 2000). In the *D. melanogaster* embryo, d*aughterless* is expressed ubiquitously and forms heterodimers with the other proneural bHLH transcription factors, so that in *daughterless* mutants the AS-C and the Atonal proteins can no longer confer proneural identity (Caudy *et al.*, 1988a; Caudy *et al.*, 1988b; Vaessin *et al.*, 1994).

Once groups of neuroectodermal cells have acquired the competence to adopt the neural fate, the neurogenic genes *Notch* and *Delta* restrict the number of cells that become neural precursors at a given time in development in *D. melanogaster* and *C. salei* by lateral inhibition (Skeath and Carroll, 1992; Stollewerk, 2002). In *D. melanogaster* loss of function of the neurogenic genes leads to an overproduction of neural stem cells due to a lack of restriction of proneural gene expression (Lehman *et al.*, 1981; Lehman *et al.*, 1983). In *D. melanogaster*, all cells of the VNE express *Notch* and *Delta* ubiquitously. When the Delta ligand binds to the Notch receptor, the

intracellular Notch domain is released and enters the nucleus, where it triggers a feedback loop resulting in the downregulation of the proneural genes, and the subsequent downregulation of Delta (Sternberg, 1993; Chitnis, 1995; Nakao and Campos-Ortega, 1996; Kidd *et al.*, 1998; Schroeter *et al.*, 1998; Struhl and Adachi, 1998). This linkage of production of ligand to the activation of the receptor leads to the amplification of small differences between cells within a proneural cluster, allowing a single neural precursor cell to arise from each competence group (Heitzler and Simpson, 1991; Sternberg, 1993; Chitnis, 1995; Marin-Bermudo *et al.*, 1995).

In the spider *C. salei*, groups of cells, rather than single cells, are selected to become neural precursors via the Notch-Delta signalling pathway. Two *Delta* and one *Notch* homologs have been identified in the spider. While *CsDelta1* is expressed exclusively in neural precursors, *CsDelta2* transcripts are present in all neuroectodermal cells (Stollewerk, 2002). However, in contrast to *D. melanogaster Delta*, expression of *CsDelta2* is up regulated in neural precursors. In addition, *CsNotch* is expressed in all neuroectodermal cells, but stronger expression was observed in areas where the first invagination sites will arise. Thus there is a dynamic modulation of *Delta* and *Notch* expression in the VNE of the spider, which, together with the functional data, indicates that lateral inhibition can restrict neural identity to a group of cells in the same manner as to a single neuroblast (Stollewerk, 2002).

Once neuroblasts in the *D. melanogaster* neuroectoderm have been selected to become neural precursors, each precursor acquires a unique cell identity based on its position in the hemisegment (Doe, 1992; Doe and Technau, 1993; Goodman and Doe, 1993; Bossing *et al.*, 1996; Schmidt *et al.*, 1997). Before the neuroblasts are selected, members of two groups of genes are expressed in each proneural cluster, which ensure that each neuroblast acquires a unique fate.

The first group, encompassing the segment polarity genes, is expressed in dorso-ventral rows of cells and enables neuroblasts that develop in different antero-posterior positions to acquire different cell fates (Bhat, 1999 and references therein). The second group, consisting of the homeobox genes *muscle segment homolog* (*msh*), *intermediate neuroblasts defective* (*ind*) and *ventral neuroblasts defective* (*vnd*), is expressed in three adjacent columns of cells in each hemisegment in lateral, intermediate and medial domains with respect to the ventral midline. The expression of these genes corresponds to the positions of the first two neuroblast populations and is necessary to create

neuroblast diversity along the dorso-ventral axis (Skeath *et al.*, 1994; Jimenez *et al.*, 1995; Isshiki *et al.*, 1997; McDonald *et al.*, 1998; Weiss *et al.*, 1998).

The combination of the antero-posterior and the dorso-ventral patterning genes creates a Cartesian coordinate system within each hemisegment, in which each proneural cluster is prefigured by a specific pattern of gene activity. This pattern is unique at a given position, allowing neuroblasts that develop in identical positions in different hemisegments to acquire the same fate. Mutations in genes of either of these groups lead to defects in fate specification: neuroblasts fail to display their normal gene expression and/or cell division profiles and produce incorrectly specified neural progeny (Goodman and Doe, 1993; Skeath, 1999 and references therein).

The action of the antero-posterior and dorso-ventral patterning genes results in neuroblasts that express unique combinations of molecular markers, allowing for the identification of each individual neural precursor in *D. melanogaster* (Doe, 1992; Doe and Technau, 1993; Goodman and Doe, 1993; Bossing *et al.*, 1996; Schmidt *et al.*, 1997). For example, a subset of neuroblasts expresses *engrailed*, which enabled the identification of the same number of neural stem cells in similar positions in the hemisegment of a variety of crustaceans, supporting the hypothesis that insect and crustacean neuroblasts are homologous (Duman-Scheel and Patel, 1999). Thus the analysis of gene expression is useful for comparison of neural structures between taxa.

While some genes allow for the identification of individual neural stem cells across species, others are expressed in all neuroblasts, a pattern that is described as panneural (Bier *et al.*, 1992). In the *D. melanogaster* VNE, two members of the *snail* zinc finger family, *snail* and *worniu*, have a panneural mode of expression. Together with the third *snail* gene, *escargot*, they have partially redundant functions in the formation of the CNS and the mesoderm (Ip *et al.*, 1994; Ashraf *et al.*, 1999; Ashraf and Ip, 2001). Triple mutants show severe defects in the development of both the mesoderm and the nervous system. In these mutants, the neural determinants Prospero and Numb are no longer asymmetrically segregated into GMCs upon neuroblast division and the generation of GMCs is disrupted (Doe *et al.*, 1991; Vaessin *et al.*, 1991; Knoblich *et al.*, 1995; Spana and Doe, 1995; Broadus *et al.*, 1998; Ashraf *et al.*, 1999).

In wildtype embryos of *D. melanogaster* and the locust *Schistocerca gregaria*, Prospero protein is present in a subset of neuroblasts and in every nascent GMC, and is thus a good marker for neural progeny (Broadus and Doe, 1995; Hassan *et al.*, 1997; Broadus and Spana, 1999). *Prospero* expression in *D. melanogaster* is restricted to the

development of the nervous system, but, while the Numb protein is asymmetrically segregated into GMCs, *numb* RNA is expressed ubiquitously throughout development.

Numb is involved in several developmental processes and has a general function as an endocytic protein in cellular homeostasis (Santolini *et al.*, 2000). In later stages of neurogenesis, Numb is essential for the correct differentiation of the progeny of sensory organ precursor cells in the PNS and of certain GMCs in the CNS. In *numb* mutants, sensory structures are present but often duplicated, as are specific neurons in the CNS (Uemura *et al.*, 1989; Rhyu *et al.*, 1994; Frise *et al.*, 1996; Spana and Doe, 1996). Prospero, on the other hand, is essential for correct axon outgrowth in later developmental stages (Vaessin *et al.*, 1991). In the spider *C. salei*, a *numb* homolog has not yet been identified, but both *snail* and *prospero* RNA and protein are expressed in all invagination sites, making these genes good markers for neural cell fate. In contrast to *D. melanogaster*, however, no asymmetric distribution of Prospero protein was observed in spider embryos (Weller, 2002).

Once the fate of a *D. melanogaster* GMC has been determined, it divides to produce two terminally differentiated neurons (or in some cases glia cells), which in turn can be identified by their cell body location, their axon morphology and the expression of specific neural markers (Whitington, 1996; Duman-Scheel and Patel, 1999). Of course, many genes are involved in positioning, specification, axonal pathfinding and survival of individual nerve cells. One gene that is relevant for this study is *islet*. *Islet* is a LIM-Homeobox gene that is necessary for at least two different neuronal properties in a subset of developing motor neurons and interneurons, including serotonergic and dopaminergic neurons of the *D. melanogaster* embryonic ventral nerve cord. While *islet* is not necessary for the survival of the expressing neurons, mutants show defects in axon pathfinding and fail to express their transmitter phenotype (Thor and Thomas, 1997). *Islet* is only expressed in neural progeny, and can thus be regarded as a marker for a subset of motor and interneurons.

To summarize, the molecular mechanisms that control neural development in *D. melanogaster* include genes necessary early in neurogenesis, neurogenic genes, genes that control antero-posterior and dorso-ventral patterning of the VNE and markers of cell fate specification. Only a small number of the genes involved in *D. melanogaster* neurogenesis could be mentioned here. Aside from the recent data from the spider *C. salei*, little is known about the molecular mechanisms of neurogenesis in other arthropods.

1.3. The choice of a model system

From a phylogenetic point of view, it is important to have a myriapod model system in which to study developmental processes to allow for comparison to data from insects, crustaceans and chelicerates, and in order to make general statements about arthropod development. While the monophyly of myriapods is still being debated, it is generally accepted that diplopods, symphylans and pauropods group together. In addition, diplopods are recognized as a monophlyletic group, yet little is known about their development and even less about the molecular mechanisms controlling developmental processes. One of the reasons for this is that there is no established model system in which basic developmental questions can be examined.

Diplopods are divided into three subgroups: the Penicillata, the Helminthomorpha and the Pentazonia. The choice of *Glomeris marginata* (Pentazonia) as my main study organism was based on several factors including the local availability of mature females, the relatively short time required for embryonic development and the descriptions of developmental processes provided by Dohle (1964). Since the VNE of many species of diplopods is not easily accessible because the embryos fold inwards very early in development, and because the development of few species has been described in as much detail as *G. marginata*, this was considered a good choice for studies of neurogenesis (Dohle, 1964 and personal communication). In order to confirm that data obtained from *G. marginata* is representative for diplopods, a tropical *Archispirostreptus* species (class Helminthomorpha) was used for comparison in some parts of this research.

1.4. Development of *Glomeris marginata*

This summary of the development of *G. marginata* is based on the detailed description provided by Dohle (1964). The *G. marginata* egg is between 0.7 and 0.8 mm in diameter and is surrounded by a smooth, thick chorion. Upon fertilization, the first cell cleavage divides the egg into two equal blastomeres, and further cleavages lead to the formation of cells that are of approximately equal size. Both yolk and blastoderm cells continue to divide until, after about seven days of development, an aggregation of blastoderm cells becomes visible. This region is the later ventral ectoderm of the germ

band (Dohle, 1964). The germ band is then visible as a thin layer of cells on top of the egg, which consists mostly of yolk cells. At this stage a further cell membrane has formed beneath the chorion, the vitelline membrane (Dohle, 1964 and personal observations).

Shortly after formation of the germ band (stage 1; stages after Dohle, 1964) the first five anterior segments that contribute to the head are visible: the antennal segment, the premandibular segment, the mandibular segment, the maxillar segment and the postmaxillar segment. Furthermore, three leg segments can be distinguished at this stage, followed by a posterior growth zone. After this, the embryonic segments of *G. marginata* arise sequentially. At stage 2, an additional leg segment has been formed by the posterior growth zone while, due to the formation of intersegmental furrows, the remaining segments are more clearly visible (Dohle, 1964).

At stage 3 limb buds arise on the antennal, the mandibular and the maxillar segments, as well as on the three leg segments. It is important to note that these anlagen are formed simultaneously. At the end of stage 3 limb buds are also visible on the fourth leg segment and a fifth segment has been generated by the posterior growth zone. A thickening of the cephalic lobe and the VNE can be observed at stage 4. Limb buds are now also visible on the fifth and sixth segments. At stage 5 a dorso-ventral furrow forms at the level of the postmaxillar segment, so that the embryo curves inward and the head eventually approaches the anal pads at stage 6 (Dohle, 1964). By the end of stage 6, the dorsal plates have extended to emcompass the yolk completely and the embryo is covered by a waxy cuticle.

At this point, after about three weeks of development, the embryo is ready to hatch. The head and the first three leg segments are fully developed, leg segments 4 - 6 have limb buds and leg segment 7 has formed. The first larval instar remains in the egg chamber and is not able to feed. After about a month, it moults into the second larval instar and leaves the egg chamber (Dohle, 1964).

Diplopod species used in this study

Picture 1. A *Glomeris marginata* female (left) and male (right). Males are approximately 0.8 cm long and females approximately 1.5 cm, individuals shown here are rolled to a ball. A closed egg chamber is visible in the lower left corner of the picture. An egg has been removed from the egg chamber between the two millipedes and is to the right of the male.

Picture 2. *Archispirostreptus sp.* adults are approximately 15 cms long. I counted a maximum of 110 leg pairs.

1.5. Aim

The aim of my research was to investigate the molecular and cellular events involved in neurogenesis in diplopod myriapods. Very little was known about the molecular mechanisms controlling development in myriapods; and, while the morphological processes of neural patterning have been studied in insects, crustaceans and chelicerates, no comparable data was available for myriapods. The first part of my research was to study the morphology of the VNE and the cellular processes necessary for neurogenesis in diplopods. After this, I isolated genes that were known to be involved in the development of the nervous system in other arthropods. My study of neurogenesis in myriapods was conducted with the intention of comparing the results to data available from the spider and other arthropods. This report contributes to a growing pool of information on the development and specification of the nervous system in arthropods and allows for comparison between distantly related organisms.

2. Materials and Methods

2.1. General

Solutions, media, and methods are standard and can be taken from Sambrook and Russell (2001). The solutions listed here are those that can be variable. Molecular methods listed are modifications of those established for the laboratory by W. Damen and M. Weller and the fixation, injection, staining and histology protocols are adapted from those used by A. Stollewerk.

2.1.1. Abbreviations

aa	amino acids
AS-C	Achaete Scute Complex
ASH	Achaete Scute Homolog
bp	base pairs
bHLH	basic Helix Loop Helix
BrdU	Bromodeoxyuridine, mitosis marker
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CNS	Central nervous system
DIG	Digoxygenin
DNA	Desoxyribonucleic acid
DNTP	Desoxynucleotriphosphate
EtOH	Ethanol
GMC	Ganglion mother cell
HMG	High Mobility Group
hr	hour(s)
HRP	Horseradish peroxidase
kb	kilo base (1000 nucleotides = 1 kb)
LB	Luria broth, standard bacterial growth medium
MEOH	Methanol
min	minute(s)
mRNA	messenger RNA
NZY	enriched standard bacterial medium
PCR	polimerase chain reaction
PNS	Peripheral nervous system
RAC	rapid amplification of cDNA ends
RNA	Ribonucleic acid
rpm	rotations per minute
RT	room temperature
sec	second(s)
TAE	Tris-Acetate-EDTA buffer, standard electrophoresis buffer
Taq Polimerase	*Thermus aquaticus* DNA Polimerase
tRNA	transfer RNA
VNE	Ventral neuroectoderm

2.2. Solutions

Unless otherwise noted, all solutions were made with Millipore water and autoclaved. Chemicals were analysis grade (p.A).

Hyb-B
- 50% Formamide
- 5 x SSC pH. 5.5
- H_20

Hyb-A (store at -20 for up to 3 months)
- 25 ml formamide
- 12.5 ml 20 x SSC
- 1 ml of 10 mg/ml salmon testis DNA
- 250 µl 20 mg/ml tRNA
- 25 µl 100 mg/ml heparin stock

10xPBS
- 80 g NaCl
- 2 g KCl
- 2 g KH_2PO_4
- 11.5 g Na_2HPO_4
- Fill to 1000 ml with H_2O, adjust to pH. 7.4

PBT (PBS + 0.1% Tween)
- 100 ml 10 x PBS
- 1 ml 10%Tween 20
- Fill to 1000 ml with H_2O

PBTrit (PBS + 0.1% Triton)
- 100 ml 10 x PBS
- 1 ml 10% Triton-X 100
- Fill to 1000 ml with H_2O

Phosphate Buffer pH. 7.0
- 577 ml 1 M Na_2HPO_4
- 423 ml 1 M NaH_2PO_4

20 x SSC
- 70.12 g NaCl
- 35.28 g Na-Citrate
- Fill to 400 ml with H_2O, adjust to pH. 5.5

Staining Buffer (prepare fresh)
- 20 ml 0.1 M Tris pH. 9.5
- 1 ml 1 M $MgCl_2$
- 400 µl 5 M NaCl
- 200 µl 10% Tween 20

2.3. Animal stocks

2.3.1. *Glomeris marginata* animals

Adult *G. marginata* (Myriapoda, Diplopoda) were collected in the city forest of Cologne, Germany, April to August 2002, and April to June 2003. Approximately 40 males and females were kept together in 20 x 10 cm plastic containers at room temperature for two weeks, after which they no longer produced sufficient numbers of eggs and so were returned to their collection site. The containers were lined with a moist paper towel and a mound of wet earth was made in one corner. This was covered by a layer of leaf litter, which functioned as food and as a hiding place for the animals. Spraying with water twice daily ensured high humidity and animals were transferred to new boxes at regular intervals to prevent fungi and bacteria from contaminating the eggs. Females cover their eggs with an earth shell to prevent injury and dryness. Eggs were collected daily and kept separately in petri dishes lined with moist paper towels until they reached days 6 to 12 of development.

2.3.2. *Archispirostreptus sp.* animals

Adult *Archispirostreptus* were obtained from the Aquazoo in Düsseldorf, Germany. Ten adults were kept at 28°C in large terraria filled to a depth of at least 20 cm with moist earth, and fed with potatoes, lettuce and carrots once a week. Females deposited clutches of up to 120 eggs into the earth approximately every three weeks, which were collected and kept separately in moist earth until the desired developmental stages were reached, at 11 - 13 days after eggs deposition. Eggs took approximately three weeks to hatch.

2.4. Methods

Unless otherwise noted, H_2O used was autoclaved Millipore water.

2.4.1. Dechorionization and fixation Protocols

2.4.1.1. *Glomeris marginata*

- Collect eggs in petri dish, cover in tap water.
- Remove eggs from dirt shell with light pressure from tweezers.
- Collect with plastic pasteur pipette, transfer to 2 ml *eppendorf* tube.
- Wash with water to remove most of dirt.
- Bleach for 2 min (bleach under 5%).
- Wash with tap water several times.
- Shake in 1ml Heptane, 50 µl Formaldehyde – 20 min for antibody staining, 4 hrs for in-situ hybridisation.
- Remove solution, wash several times in EtOH for antibody staining or MEOH for in-situ hybridisation.
- Freeze at –20°C over night before devitelinization with tweezers.

2.4.1.2. *Archispirostreptus sp.*

- Separate eggs from earth with a small brush.
- Wash in water in round 15 ml *Falcon* tube.
- Bleach 1 min (bleach under 5%).
- Remove, wash with tap water several times until dirt is removed.
- Remove water with a glass pipette.
- Either transfer to a 1.5 ml *eppendorf* and freeze at –80°C for RNA extraction or:

- Add 4 ml Heptane, leave on wheel overnight.
- Add 3.2 ml 1 x PBS, 800 µl Formaldehyde and shake 1 hr.
- Wash with PBS several times, dechorionate with tweezers and freeze in 100% EtOH for antibody stainings or 100% MEOH for in-situ hybridisation.

2.4.1.3. Osmium fixation of *G. marginata* embryos

- Fix eggs as described in 2.4.1.1. for antibody staining. After removal of the fixation solution, do not wash in EtOH. Instead, devitelinize in H_2O with 10% BSA.
- Wash 3 times in Phosphate Buffer.

All following steps on ice under the hood. Cool water and the EtOH solutions on ice. Collect all poisonous Osmium waste in separate container.

- Mix 284 µl Phosphate Buffer with 16 µl Glutaraldehyde *(Serva)* and 100 µl Osmium (Serva), leave embryos in this for 1 hr in the dark.
- Wash 2 x 5 min in 500 µl cold H_2O .

- Wash 1 x 10 min in 500 µl 50% EtOH.
- Wash 1 x 10 min in 500 µl 70% EtOH.
- Wash 1 x 10 min in 500 µl 80% EtOH.
- Wash 1 x 10 min in 500 µl 90% EtOH.
- Wash 1 x 10 min in 500 µl 96% EtOH.
- Wash 2 x 10 min in 500 µl 100% EtOH.

Following steps at RT under the hood

- thaw Araldit (see Stollewerk *et al.*, 1996, for solutions)
- Wash embryos 2 x 15 min in 100% Acetone, transfer to a glass dish
- Mix 50% Acetone, 50% Araldit in a separate *eppendorf* tube by pipetting up and down with a glass pipette.
- Add Acetone/Araldit to embryos in the glass dish. Make sure the embryos are covered by the solution. Cover with glass slide that does not close airtight (tape on the sides of the glass dish is useful).
- Leave overnight.

Arrange embryos in plastic forms under the microscope. Incubate for 48 hr at 70°C. Transverse sections were then cut (for methods see Stollewerk *et al.*, 1996) and photographed under a *Zeiss Axiophot* microscope.

2.4.2. Molecular methods

2.4.2.1. Agarose gels

All agarose gels used, unless otherwise specified, were made with 1% weight per volume standard agarose in 1 x TAE buffer, heated to a boil in the microwave. The solution was cooled to approximately 40°C and 3.5 µl Ethidium Bromide stock solution *(Roche)* was added before pouring into gel chambers for hardening. Large gels were run at a maximum of 140 Volt and minigels at 70 Volt provided by a *BioRad* power unit and photographed under Ultraviolet light as a record. Standard loading buffer was added to the DNA or RNA to be separated (usually at least 2 µl). Markers used were *1kb+* from *GIBCO* and *Smartladder* from *Eurogentec*.

2.4.2.2. RNA isolation

- 250 µl embryos from –80°C, cool centrifuge to 4°C.
- Mix the embryos with 750 µl *TRIZOL (Invitrogene)*.
- Crush embryos in the *TRIZOL* solution under the hood.
- Centrifuge for 10 min at 15000 rpm at 4°C.
- Transfer the supernatant to a new tube, leave at RT 5 min.
- Add 20 µl Chloroform, mix well, leave at RT 15 min.
- Centrifuge for 15 min at 15000 rpm at 4°C.

- Transfer the supernatant to a new 1.5 ml *eppendorf* tube.
- Add 500 µl 100% Isopropanol, mix, leave at RT 10 min.
- Centrifuge for 15 min at 15000 rpm at 4°C.
- Remove supernatant on ice.
- Wash with 500 µl cold 70% EtOH.
- Centrifuge for 10 min at 15000 rpm at 4°C.
- Remove supernatant, dissolve RNA in 50 µl H_2O.
- Check 1 µl on an agarose gel and measure RNA content with a photometer.

For the *Marathon RACE* template, poliadenylated RNA was isolated using the *PoliATtract kit (Promega)*.

2.4.2.3. PCR templates

CDNA was synthesised by reverse transcribing RNA extracted from 7 to 12 day *G. marginata*, or 2 to 3 week *Archispirostreptus* embryos, respectively, using the *Superscript III kit (Invitrogene*). Templates for the rapid amplification of cDNA ends were constructed for both species using the *GeneRacer Kit (Invitrogene)*, and for *G. marginata* a second RACE template was made using the *Marathon cDNA amplification kit (Clonetech)*.

2.4.2.4. Primers

In the case of *GmASH*, *GmNotch*, *GmDelta, AsAsh,* and *AsDelta* degenerate primers for the respective genes were used as described in Stollewerk *et al.*, 2001 and Stollewerk, 2002. For the AS-C homologs, more specific degenerate primers were designed based on the available invertebrate and vertebrate sequences. However, no further fragments were identified. The degenerate primers I designed to isolate my initial fragments are listed below, along with the annealing temperatures used for PCR reactions. Degenerate primers were designed by hand based on alignments of conserved sequences from the *NIH* Blast database made in *BioEdit*. Generally, two forward (F) and two reverse (R) primers were designed for each gene (PCR1), with the second pair of primers lying within the fragment amplified by the primary primers (PCR2), so that a second so-called nested PCR could be performed using the products of the first as a template. Melting temperatures (T_M) were calculated for each primer. The following formula provided by *metabion* was used to ensure that the temperature for the forward and the reverse primers were close.

$$T_M = (\#GC/\ \#\ \text{nucleotides} \times 0.41 + 69.31) - 650/\#\ \text{nucleotides}$$

In the case of highly degenerate primers the average of the maximum and the minimum primer temperature was used. RACE primers were designed on the same principle, except that the annealing temperatures were selected to match that of the adaptor primer. Primers were ordered from *metabion* lyophilised and then dissolved in H_2O to a concentration of 100 pmol/µl.

Table 1. The amino acid sequence shown in the column marked "aa" is the *D. melanogaster* sequence, unless otherwise noted. The column termed "use" gives the PCR reaction in which the primer was used, the orientation and the temperature. The column termed "°C" gives the melting temperature of the primer as calculated by metabion. The column "#" gives the primer's number as a reference for future experiments. The *ASH* primers are based on the bHLH domain, and since there are several members of this family in *D. melanogaster*, the aa sequence noted here is the consensus sequence I used for primer design. The *asense* primers are the only ones that did not lead to a correct fragment. As no *asense* homologs have been identified outside of insects it is possible that this gene is not highly conserved. In the case of the second set of *atonal* primers (*atonal spec*), an alignment of the one fragment obtained with the first set with *Lithobius forficatus* nucleotide sequences (provided by D. Kadner) led to a more specific primer pair for the same region.

Gene	aa	Primer sequence (5'-3')	use	#	°C
ASH vert (ASH)	A/S V A R R N E	GCS GTG GCV CGS CGS AAC GAG	PCR1/F 61°C	57	70.9
ASH vert (ASH)	L R I/M/L A/V V E Y I	GAT GTA CTC SAC GGC NGA NCG CAG	PCR1/R 61°C	59	67.8
ASH vert (ASH)	R N E R E R N	CGS AAC GAG CGV GAG MGV AAC	PCR2/F 60°C	58	65.4
ASH vert (ASH)	V D/E T L R I/M/L A	GCS GAN CGC AGH GTB TCC AC	PCR2/R 60°C	60	64.5
ASH	R R N E R E R	MGV MGV AAY GMB MGN GAR CG	PCR1/F 55°C	61	62.4
ASH	T L R I/M/L A/V V E	YTC NAC NGC VRW NCG YAG YG	PCR1/R 55°C	63	61.7
ASH	N E R E R N R	AAY GMB MGN GAR CGN AAY CG	PCR2/F 55°C	62	59.7
ASH	V D/E T L R I/M/L A/V	GCC GAN CGB ARH GTN TCN AC	PCR2/R 55°C	64	61.4
asense (as)	Q V N N G F A	CAR GTN AAY AAY GGN TTY GC	PCR1/F 53°C	171	55.3
asense (as)	P G T N T Y Q	TGR TAN GTR TTN GTN CCN GG	PCR1/R 53°C	173	57.3
asense (as)	V E T L R M A	GTN GAN ACN YTN MGN ATG GC	PCR2/F 53°C	172	58.3
asense (as)	Q Y I R I R I P G	CCN GGD ATN CKN ATR TAY TG	PCR2/R 53°C	174	55.9
atonal (at)	A A N A R E R	GCN GCN AAY GCN MGX GA	PCR1/F 54°C	149	56.3
atonal (at)	E T L Q M A Q	TGN GCC ATY TGN ARN GTX TC	PCR1/R & 2/R	148	58.3
atonal (at)	A R E R R R M	CGN GAR MGN MGN MGX ATG	PCR2/F 53°C	150	57.2
atonal spec (at)	bHLH domain	GAG AGG AGG AGG ATG MAC AG	PCR1/F 58°C	155	60.4

Gene	aa	Primer sequence (5'-3')	use	#	°C
atonal spec (at)	bHLH domain	TGG GCC ATT TGH AGB GTG TCG	PCR1/R 58°C	156	61.8
atonal spec (at)	bHLH domain	GGA TGM ACA GTT TRA AYG TWM GC	PCR2/F 56°C	157	58.9
atonal spec (at)	bHLH domain	TGH AGB GTG TCG TAY TTD GAC	PCR2/R 56°C	158	57.6
daughterless (da)	R R Q A N N A	MGN MGN MWR GCN AAY AAY GC	PCR1/F 56°C	167	58.3
daughterless (da)	P K A A C L K	TTN ARR CAN GCN GCY TTN GG	PCR1/R 56°C	169	58.3
daughterless (da)	I R D I N E A	ATH MGN GAY ATH AAY GAR GC	PCR2/F 52°C	168	53.6
daughterless (da)	E Q Q V R E R	CKY TCN CKN ACY TGY TGY TC	PCR2/R 52°C	170	59.4
Dichaete/Sox (di)	H I K R P M N A	RTN AAR MGN CCN ATG AAY GC	PCR1/F 54°C	144	56.3
Dichaete/Sox (di)	E H P D Y K Y R	GKR TCY TTR TCN GGR TGY TC	PCR1/R 54°C	147	58.3
Dichaete/Sox (di)	M N A F M V W	ATG AAY GCN TTY ATG GTX TGG	PCR2/F 53°C	145	55.0
Dichaete/Sox (di)	H M K E H P	TCN GGR TGY TCY TTC ATR TG	PCR2/R 53°C	146	56.3
ind	T A F T S T Q	ACN GCN TTY ACN WSN ACN CA	PCR1/F 55°C	179	57.3
ind	W F Q N R R V K	TTN ACN CKN CKR TTY TGR AAC C	PCR1/R 55°C	181	58.4
ind	L E L E R E F	YTN GAR YTN GAR MGN GAR TT	PCR2/F 51°C	180	54.2
ind	E K Q V K I W	CCA DAT YTT NAC YTG YTT YTC	PCR2/R 51°C	182	53.6
islet	L E W H A A C	YTN GAR TGG CAY GCN GCN TG	PCR1/F 56°C	163	61.4
islet	P A F Q Q L V	ACN ARY TGY TGR AAN GCN GG	PCR1/R 56°C	165	58.3
islet	R D G K T Y C	MGN GAY GGN AAR ACN TAY TG	PCR2/F 55°C	164	56.3
islet	Q P P W K A L	ARN GCY TTC CAN GGN GGY TG	PCR2/R 55°C	166	61.4
msh	N R K P R T P	AAY MGN AAR CCN MGN ACN CC	PCR1/F 53°C	175	59.4
msh	E K I K M A A	GCN GCC ATY TTN ADY TTY TC	PCR1/R 53°C	177	54.9
msh	T P F T T Q/A Q	ACN CCN TTY ACN ACN CAR CA	PCR2/F 53°C	176	57.3
msh	Q E A E I E K	TTY TCD ATY TCN GCY TCY TG	PCR2/R 53°C	178	54.9
numb	S S K P H Q W Q	CNW SNM RNC AYC ART GGC A	PCR1/F 54°C	132	57.8
numb	D K E C G V T M	GTN ACN CCR CAY TCY TTX TC	PCR1/R 54°C	134	56.3
numb	V D Q T I E K V	GTN GAY CAR ACN ATH GAR AAR	PCR2/F 56°C	133	58.2
numb	H A V G C A F A	AAN GCR CAN CCN ACX GCR TG	PCR2/R 56°C	135	60.0
pannier (pan)	E C V N C G A	GAR TGY GTN AAY TGY GGN GC	PCR1/F 56°C	159	59.4
pannier (pan)	A M R K D G I Q	ATN CCN TCY TTN CKC ATN GC	PCR1/R 56°C	160	57.3
pannier (pan)	R D G T G H Y	CGN GAW GGN ACN GGN CAW TA	PCR2/F 57°C	161	59.4
pannier (pan)	H G V N R P L	ARN GGN CKR TTN RSN CCR TG	PCR2/R 57°C	162	60.4

Gene	aa	Primer sequence (5'-3')	use	#	°C
prospero (pros)	H L R K A K L M	YTN MRN AAR GCN AAR YTX ATG	PCR1/F 48°C	140	53.0
prospero (pros)	V P E Y F K S	TWY TTR AAD WWY TCN GGX AC	PCR1/R 48°C	141	49.8
prospero (pros)	E F F Y I Q M E	GAR TTY TWY TAY ATH CAR ATG G	PCR2/F 50°C	142	52.5
prospero (pros)	R E F F R A I	TDN CGN YKR AAR AAY TCX C	PCR2/R 50°C	143	52.2
snail	G A L K M H I R	TNG GNG CNY TNA ARA TGC A	PCR1/F 52°C	136	54.5
snail	Q T H S E V K	TYT TNA CNT CNG WMT GNG TYT G	PCR1/R 52°C	138	57.5
snail	G K A F S R P W	AAR GCN TTY WSN MGN CCX TGG	PCR2/F 56°C	137	59.8
snail	A F A D R S N	RTT NSW NCK RTC NGC RAA XGC	PCR2/R 56°C	139	58.8
vnd/tinman	E R R F R Q Q	GAR MGN MGN TTY MGX CAR CA	PCR1/F 54°C	151	57.3
vnd/tinman	V R N G K P C	CAN GGY TTC CNR TCN CKX AC	PCR1/R 54°C	154	59.4
vnd/tinman	S A P E R E H	AGC NCC NGA RMG NGA RCA	PCR2/F 56°C	152	58.2
vnd/tinman	V A V P V L V	ACN ARN ACN GGN ACN GCX AC	PCR2/R 56°C	153	58.4

Table 2. The expected size of fragments amplified by the nested PCR.

Gene	*ASH*	*at*	*as*	*da*	*di*	*ind*	*islet*	*msh*	*numb*	*pan*	*pros*	*snail*	*vnd*
bp	150	150	350	150	200	100	770	160	150	180	180	120	190

2.4.2.5. PCR reactions

RACE PCR reactions were performed as directed in the kit manuals. For RT-PCR the following protocols were used:

Primary PCR
2 µl cDNA
3 µl DNTP (2 mM, *Amersham*)
3 µl PCR buffer (with the polimerase, *Applied Biosystems*)
1.5 µl primer 1 (20 pmol/µl)
1.5 µl primer 2 (20 pmol/µl)
0.5 µl Taq polimerase (*AmpliTaq*, *Applied Biosystems*)
18.5 µl H_2O
30 µl Total volume. In 0.5 µl *eppendorf* tubes, in *eppendorf* cycler.

Nested PCR
1 µl Primary PCR
3 µl DNTP (2 mM, *Amersham*)
3 µl PCR buffer
1.5 µl primer 1 (20 pmol/µl)
1.5 µl primer 2 (20 pmol/µl)

0.5 µl Taq polimerase (*AmpliTaq*, *Applied Biosystems*)
19.5 µl H_2O
30 µl Total volume. In 0.5 µl *eppendorf* tubes, in *eppendorf* cycler.

PCR Program on an *eppendorf* Mastercycler

Heat lid to 105°C

1. 1 min 94°C
2. 30 sec 94°C
3. 1 min Annealing Temperature ($T_A = T_M - 2$)
4. 90 sec Elongation time
5. Repeat steps two to four 35 times
6. 5 min 72°C

   Hold 4°C

Separate 5 µl on an agarose gel. For cloning, the nested PCR was repeated to obtain a larger volume.

2.4.2.6. Preparation of vectors

- Mix in a 1.5 µl *eppendorf* tube:

1 µl	*pZero* stock (*Stratagene*)
1 µl	EcoRV restriction enzyme for blunt ends (*Roche*)
1 µl	Buffer provided with enzyme
7 µl	H_2O
10 µl	Total volume

- Leave at 37°C for 30 min.
- Add one volume of phenol-chloroform (50 µl), vortex, centrifuge 5 min at maximum speed.
- Transfer the supernatant to a new tube, add one volume of chloroform (50 µl), vortex, centrifuge 5 min at maximum speed.
- Transfer the supernatant to a new tube, add 5 µl Sodium acetate (3 M, pH 5.2), mix.
- Add 110 µl 100% EtOH, leave at –20°C at least one hr.
- Centrifuge at maximum speed for 30 min.
- Carefully remove liquid, add 500 µl 70% EtOH, centrifuge 15 min at maximum speed.
- Remove liquid, dry for 5 min at 37°C. Dissolve in 90 µl H_2O, store at –20°C.
- Separate 1 µl on an agarose gel to check the size.

2.4.2.7. Preparation of inserts

To ensure that the insert has blunt ends, the PCR is treated with Kleenow (*Roche*).

51 µl PCR reaction
1 µl Kleenow (*Roche*)
6 µl ligation buffer (*Roche*)
2 µl DNTPs (2 mM, *Amersham*)
60 µl Total volume

Leave at 37°C for 40 min

Transfer to an elution gel (0.7% agarose gel with bigger slots) and run at maximally 70 Volt for approximately two hrs. Cut out gel slice of fragment of interest, elute using the *MiniElute kit* (*Quiagen*) and dissolve in 10 µl of H_2O.

2.4.2.8. Ligation

10 µl fragment
2 µl ligation buffer (*Roche*)
1 µl Cut *pZero* vector (section 2.4.2.6.)
6 µl H_2O
1 µl ligase (*Roche*)
20 µl Total volume

Leave at 16°C in a PCR machine for 1 to 10 hrs (depending on the size and concentration of the fragment). Store at –20°C until use.

2.4.2.9. Electroporation

Top 10 cells (*Invitrogene*) were made electrocompetent using the protocol provided by Invitrogene. The following protocol was used for electroporation:

- Cool an electrocuvette (*BioRad*) on ice.
- Thaw NZY+ medium (stored in 15 ml tubes at –20°C).
- Set the electroporation device (*BioRad*) to 1.8 milliVolts per second.
- Thaw one tube of electrocompetent cells on ice.
- Add up to 2 µl ligation, mix gently with pipette tip. Transfer to electrocuvette.
- After one pulse, add 200 µl NZY medium to the cuvette, and transfer this together with the bacteria to a test tube.
- Shake for 1 hr at 37°C.
- Plate on LB-Agar plates (with 0.1% Kanamycin 50 mg/ml), grow overnight at 37°C.

2.4.2.10. Colony PCR

In order to ensure that the plasmids selected for further amplification contained an insert of the correct size, a so-called colony PCR was performed. Usually, at least 24 colonies per insert were amplified, so the protocol given here is for 24 but it can be increased to 96 without an increase in the amount of Taq polimerase used (*Amplitaq, Applied Biosystems*; any Taq polimerase can be used and the protocol adjusted accordingly). The PCR reaction was mixed together and 10 µl distributed into each of the 24 0.5 µl *eppendorf* tubes on ice. Then, one bacterial colony was picked from the plate with a toothpick, dipped into the PCR reaction and then streaked onto a so-called replica plate

into a numbered field to allow for the individual identification of each colony amplified. The replica plate was left at 37°C until needed, then stored at 4°C. The PCR was run, separated on a gel, and the colonies containing an insert of the correct size could be picked from the replica plate and amplified.

The standard PCR program (see section 2.4.2.5.) was used with an annealing temperature of 60°C. The PBsA and PBsE primers are vector primers listed as such in the vector map for *pZero* (*Stratagene*) and were used at a concentration of 20 pmol/µl.

PCR protocol for 24 reactions:

24 µl PCR Buffer (*Applied Biosystems*)
24 µl DNTPs (2mM, *Amersham*)
12 µl PBsA primer
12 µl PBsE primer
1 µl Taq Polimerase (*Amplitaq, Applied Biosystems*)
167 µl H_2O
240 µl Total volume, 10 µl per tube.

2.4.2.11. Plasmid preparations (Minipreps)

- Grow overnight cultures at 37°C shaking: 3 ml LB and 30 µl Kanamycin (50 mg/ml), inoculate one test tube with 1 colony.

Next day:

- Prepare Heating block at 95 - 100°C
- Tris EDTA LiCl Triton buffer (so-called TELT buffer, always make fresh)

Ingredients (in µl)	**12x**	**24x**	**36x**	**48x**	**96x**
1 M Tris pH. 7.5-8	100	200	300	400	800
0.5 M EDTA pH. 7.5-8	260	520	780	1,040	2,080
10% Triton	80	160	240	320	640
3,2 M LiCl	1,560	3,120	4,680	6,240	12,480
Lysozym 50 mg/ml	200	400	600	800	1,600
Rnase A 10 mg/ml	6	12	18	24	48
Total Volume	**2206**	**4412**	**6618**	**8824**	**17,648**

- Spin down cells (2 min 6000 rpm) in 1.5 ml *eppendorf*, remove supernatant, add remaining cells, spin down and remove supernatant.
- add 165 µl TELT buffer to each eppi, resuspend the pellet (by vortexing).

- 5 min at RT (vortex for 5 min).
- 2 min at 95 - 100°C.
- 5 min on ice.
- centrifuge 10 min at maximum speed.
- remove snot pellet with a toothpick, discard.
- add 165 µl 100% Isopropanol, mix.
- centrifuge 30 min at maximum speed.
- remove most of the supernatant, discard. Wash the pellet with 500 µl 70% EtOH.
- centrifuge at maximum speed 15 min.
- remove most of the EtOH, let the rest evaporate at 37°C.
- dissolve pellet in 70 µl H_2O.

For *Sephadex* (*Amersham*) step:
- put 750 µl *Sephadex* (10 g in 150 ml water, autoclave, store at 4°C after opening) onto Sephadex column. Centrifuge 2 min at 4000 rpm.
- place column in new *eppendorf* tube (marked), pipette the solution onto middle of column. Centrifuge 2 min 4000 rpm.
- check 2 µl on agarose gel. Use 2 µl for Sequence reaction. Store at –20°C.

2.4.2.12. Sequencing and analysis

Sequence reaction

2 µl	Template (0.1 - 0.25 µg/µl)
1.5 - 2 µl	*Big Dye* (*Applied Biosystems*, version 2.0 or 3.0)
2 µl	Primer 3 pmol/µl (either vector primer or sequence specific primer)
4 - 4.5 µl	H_2O
10 µl	Total volume

Program on an *eppendorf* Mastercycler:
Heat Lid to 105°C

1. 96°C 1 min
2. 96°C 10 sec
3. 40°C 15 sec
4. 60°C 4 min
5. Repeat steps 2 - 4 30 x
6. Hold at 4°C

***Sephadex* for Sequencing:**
- After sequence reaction is complete, add 10 µl H_2O, repeat the *Sephadex* step (see section 2.4.2.11.).
- Dry sequence reaction in a vacuum manifold for 30 min.

Sequences were read on the automatic *ABI* Sequencers in the sequencing facility at the Institute for Genetics of the University of Cologne.

Sequence Analysis

Sequences were analysed and aligned with related amino acid sequences taken from the *NIH* Blast database in *Bioedit*. Trees were constructed using the *PAUP* NJ minimum evolution algorithm with 1000 bootstrap replicates. Positions where an amino acid insertion was present in only one sequence were removed, as was the variable part of the loop for the ASH and the Atonal alignments. Since the portions of these two genes that could be aligned were very short (the bHLH domain), the presence or absence of a loop was used as an extra character (32/57 informative characters for tree 1, 32/47 for tree 2). For the Sox tree, the corresponding HMG regions of other proteins were used (37/49 informative characters). For Delta, the DSL domain and EGF repeats 1 and 2 were aligned (70/109 informative characters), for Notch the 5´ sequences up to EGF repeat 12 were aligned (266/311 informative characters). In the case of the Snail, Prospero and Numb trees, conserved motifs (as described in section 3.3.4.) containing 43/98, 82/162 and 54/154 informative characters, respectively, were aligned. For the Islet tree, all gaps were removed (51/153 informative characters).

2.4.3. Injection of *G. marginata* embryos

Several methods were attempted to inject *G. marginata* embryos during the breeding seasons in 2002 and 2003. The standard methods used for flies and for the spider *Cupiennius salei* did not work because the *G. marginata* embryos need very high humidity to survive (methods for *C. salei* described in Schoppmeier and Damen, 2001). Injections on various agar substrates were equally problematic. Ultimately it was found that injection of embryos as soon as the germ band was visible and the viteline membrane was fully developed was possible using the protocols presented in this section. The youngest embryos to survive using this method were stage 2 upon injection. However, injections with double-stranded RNA were not successful, at least no changes in gene expression patterns could be observed by in-situ hybridisation.

It will be necessary to develop new methods to access younger embryos for functional studies; initial experiments with electroporation were unsuccessful, but with some modification they may work. The injection method described here led to good results with BrdU and is thus in principle functional.

2.4.3.1. Preparation of embryos

- Use 5 - 7 day eggs .
- Collect eggs in petri dish, cover in tap water.
- Remove eggs from dirt shell with light pressure from tweezers.
- Collect with plastic pasteur pipette, transfer to 2 ml *eppendorf* tube.
- Wash with water to remove most of dirt.
- If the culture was infested with fungi, rinse once with bleach (under 5%) and then wash in water.
- Remove chorion by hand with fine tweezers under the microscope in tap water, discard damaged eggs.
- Transfer eggs to a petri dish filled with water that has a glass microscope slide in the middle so the eggs can be oriented for injection.

2.4.3.2. Preparation of capillaries

Microinjection needles were prepared from borosilicate glass capillaries with a filament (outer diameter 1 mm, inner diameter 0.580 mm; from *Hilgenberg*, Malsfeld, Germany) using a *Sutter* Micropipette puller (Heat 480, Pull 80, Vel 100, Time 150) For injection the needles were backloaded with Bromodeoxyuridine (BrdU, *Roche*) at a concentration of 1:50 diluted with tap water and a 0.5 µl phenol red (P-0290, *Sigma*) that is used as a visible marker for injection.

2.4.3.3. BrdU injections

Microinjections were performed on a *Zeiss* dissection microscope equipped with an *eppendorf* Micromanipulator *5171* connected to an *eppendorf Femto-Jet* microinjection unit. The pressure and the injection time were adjusted to the size of the injection needle as was estimated by the visible amount of phenol red. *G. marginata* eggs were injected at an angle of approximately 45 degrees above the surface of the egg and the solution was injected into the space between the embryo and the viteline membrane.

2.4.3.4. Care of eggs after injection

Embryos were disturbed as little as possible, and were left in petri dishes filled with tap water at RT in the dark. The tap water was changed every day to the extent possible and any damaged eggs removed. Embryos developed normally and, if left long enough,

even hatched in water. For BrdU staining, eggs were fixed after one day following the protocol 2.4.1.1. for antibody staining and then stained according to protocol 2.4.4.5.

2.4.4. Staining and histology

2.4.4.1. DIG-labeled RNA probes

The insert of interest with the RNA Polimerase binding sites attached was first amplified via PCR and then reverse-transcribed.

PCR amplification
1 µl Plasmid preparation
10 µl PCR buffer (*Applied Biosystems*)
10 µl DNTPs (*Amersham*, 2 mM)
5 µl PBsE (20 pmol/µl)
5 µl PbsA (20 pmol/µl)
68 µl H_2O
1 µl Taq polimerase (*Amplitaq, Applied Biosystems*)
100 µl Total volume, distribute 25 µl per tube to four PCR tubes.

For PCR program see section 2.4.2.11.

After completion of PCR:

- Clean PCR product with the *QuiaQuick PCR purification kit* (*Quiagen*) .
- Dissolve in 30 µl H_2O.
- Separate 0.5 µl on an agarose gel with *Smartladder* (*Eurogentec*) to estimate the concentration. .
- The choice of an RNA polimerase depends on the cloning vector and the orientation of the insert, DIG-labeled probes should be in a 3' to 5' (so-called antisense) direction so that they can bind to the endogenous mRNA in the cell.

In-vitro transcription
500 ng template
1 µl DIG NTPs (*Roche*)
1 µl RNAse-inhibitor (*Roche)*
1 µl Transcription buffer (*Roche*)
1 µl RNA polimerase (*Roche*)
Add H_2O to a final volume of 10 µl

Leave at 37°C for 2 hrs.

- Add: 1 µl tRNA (*Roche*)
  1 µl EDTA (0.5 M, pH. 8)
  100 µl H_2O
- Use an *Ultrafree* Column (*MC 100 000 NWL Filter unit*, *Millipore*).
- Equilibrate by centrifuging once at 6000 rpm for 5 min with 100 µl H_2O.
- Add probe, centrifuge 5 min at 6000 rpm.

- Repeat 2 x with 100 µl H_2O.
- Add 100 µl H_2O, pipette up and down and transfer to a clean 1.5 ml *eppendorf* tube.
- Control 5 µl on a gel, store the rest at –80°C.

Or: Use other columns (*Montage PCR centrifugal filter devices*, *Millipore*) for the centrifugation step instead of the *Ultrafree*. In this case, an extra 300 µl H_2O are added and the columns are centrifuged for 15 min at 2000 rpm, then turned over and the probe is eluted into 50 µl H_2O by centrifuging again for 15 min at 2000 rpm. Again, separate 5 µl on a gel, store the rest at –80°C.

2.4.4.2. Whole mount in-situ hybridisation

Use approximately 30 embryos per reaction. All steps are in 2 ml *eppendorf* tubes. All steps on shaker or wheel, and 1 ml of the solution used unless otherwise noted. Preheat waterbath to 65°C before starting.

Remove the viteline membrane with tweezers in MEOH in a glass dish under the microscope.

Day 1
- 5 min 50% MEOH
- 5 min 30% MEOH
- 5 min PBT
- 20 min postfix in 1 ml PBT, 140 µl Formaldehyde
- 5 min PBT
- 5 min PROTEINASE K (*Roche*; 2.5 µl 1:10 dilution in 1 ml PBT)
- 5 min PBT
- 20 min postfix in 1ml PBT, 140 µl Formaldehyde
- 5 min PBT
- 5 min PBT
- 5 min H_2O
- 60 min 1 ml TEA (1.2 M Trietholamine, pH. 7, *Sigma*)**,** 2.5 µl Acete Anhydrid (*Sigma*)
- 5 min PBT
- 5 min PBS
- 5 min Hyb B
- 4 hrs Hyb A 65°C
- 20 ul Hyb A + probe overnight at 65°C (probe concentration is variable but 5 µl is usually sufficient)

Day 2
<u>At 65°C:</u>
- remove probe
- 15 min Hyb B + 25% 2 x SSC (pH 5.5)
- 15 min Hyb B + 50% 2 x SSC
- 15 min Hyb B + 75% 2 x SSC
- 15 min 2 x SSC
- 30 min 0.2 x SSC

- 30 min 0.2 x SSC

Room Temperature:

- 10 min 0.2 x SSC + 25%PBT
- 10 min 0.2 x SSC + 50%PBT
- 10 min 0.2 x SSC + 75%PBT
- 10 min PBT
- Block 2 hrs in PBT + 2% Sheep Serum (*Sigma*)
- Antibody overnight 1:2000 in PBT at 4°C

Day 3

Wash 8 x 15 min PBT
Wash 2 x 5 min in Staining Buffer

Stain in 1 ml Staining Buffer with 4.5 µl NBT and 3.5 µl X-phosphate (both *Roche*) in the dark. Stop by washing with PBT several times and then fixing in 1 ml PBT and 140 µl Formaldehyde on the shaker for 20 min. Replace this by 100% MEOH and store at –20 °C.

2.4.4.3. Phalloidin-rhodamin staining

Important: Embryos used for this staining should not have any contact with Tween 20 or MEOH, otherwise the staining will be unsuccessful.

- Use embryos that have been at –20°C in ETOH.
- Remove as much of the viteline membrane as possible with tweezers in a glass .
- Block for at least 1 hr in 900 µl 1 x PBS and 100 µl 10% BSA in the dark on wheel or shaker.
- Dry 10 µl of Phalloidine stock (*Molecular Probes*) in a vacuum manifold for 10 min to remove MEOH, dissolve the pellet in 180 µl 1 x PBS and 20 µl 10% BSA (*Sigma*).
- Stain in the dark for at least one hr upright on a wheel or shaker, wash.
- Make flat preparations in 50% Glycerine (dilute with H_2O) and examine under the confocal laser scanning microscope (LSM).

2.4.4.4. Antibody staining for HRP

HRP antibody from *Jackson Immunoresearch* (#323-005-021; Affinipure Rabbit Anti-HRP)

- Embryos in 100% MEOH devitelinize.
- Wash 3 x 15 min in PBSTrit.
- Block 2 hrs in 2% goat serum with PBSTrit.
- Antibody Rabbit anti-HRP 1:1000 in blocking solution overnight at 4°C on the wheel.

Light microscope:

- Wash 1.5 hrs in PBSTrit.
- Staining buffer with Triton instead of Tween, 2 x 5 min, wash, stain n the dark in 1 ml buffer with 20ul NBT/BCIP mix (*Roche*).

Confocal microscope:
- Wash 2 hrs in PBSTrit with 2% goat serum.
- Secondary antibody goat anti rabbit Cy5 conjugated (dilution 1:1000) in PBSTrit with 2% goat serum.
- Wash 1.5 hrs in PBSTrit.
- Make flat preparations in 50% Glycerine (dilute with H_2O) and examine under the LSM.

2.4.4.5. Double staining: Anti-Phospo-Histone 3 and Phalloidin-rhodamin

The Anti-Phospho-Histone 3 (PH3) antibody was provided by F. Sprenger (Institute for Genetics, Cologne).

- Embryos in 100% ETOH devitelinize.
- Wash 3 x 15 min in PBS.
- Block 2 hrs in PBS with 10% BSA.
- PH3 antibody (dilution 3: 1000) in blocking solution overnight at 4°C on the wheel.
- Wash 2 hrs every 10 min in PBS with 2% Goat serum.
- Antibody 2 for 2 hrs RT in PBS 2% goat serum (dilution 2.5: 100) Cy5 conjugated anti-rabbit in the dark.
- Wash 1.5 hrs in PBS in the dark.
- Dry 10 µl of Phalloidine stock (*Molecular Probes*) in a vacuum manifold for 10 min to remove MEOH, dissolve the pellet in 180 µl 1 x PBS and 20 µl 10% BSA (*Sigma*).
- Stain in the dark for at least one hr upright on a wheel or shaker.
- Wash 1 x PBS.
- Make flat preparations in 50% Glycerine (dilute with H_2O) and examine on the LSM.

2.4.4.6. BrdU staining

G. marginata embryos were labeled with BrdU (*Roche*) via injection as described in section 2.4.3. Alternately, the following protocol was used. Eggs were washed with water, dechorionized by hand, and then a small hole was made in the viteline membrane with a glass capillary. The embryos were transferred to a 2 ml *eppendorf* tube and left in 490 µl tap water and 10 µl BrdU (*Roche*) for 20 - 90 min. After this time they were fixed using the protocol in 2.4.1.1. for antibody staining. Before detection of BrdU (*Roche*), embryos were devitelinized by hand.

To detect the labeled cells, the BrdU labelling kit was used (*Roche*). Stained embryos were either prepared and photographed as whole mounts using the *Zeiss Axiophot* microscope or fixed with osmium following the protocol 2.4.1.3. for transverse sections.

3. Results

3.1. *Glomeris marginata* as a study organism

As with any non-standard organism, the first step to examining the mechanisms of neurogenesis in *G. marginata* was to establish protocols for egg collection, fixation, antibody staining, in-situ hybridization and injection of embryos. It was found that some procedures, such as egg collection and flat preparation of stained embryos, are time-consuming. However, embryos survive well to the desired stage, large numbers of eggs can be collected for DNA and RNA isolation and it is possible to obtain in-situ and antibody stainings with very little background. After many failed attempts to inject *G. marginata* embryos in various standard solutions and media, it was found that they can be injected and survive with no difficulties in normal tap water without additional supplements. To summarize, the advantages of using *G. marginata* as a study organsim are that it can be cultured in the lab, eggs are abundant during the breeding season and it was possible to establish standard protocols for the study of developmental processes. The main disadvantage is that eggs are only available during the breeding season from April to the end of June.

A tropical *Archispirostreptus* species was used to ensure that data obtained from *G. marginata* is representative of diplopods. Laboratory cultures were established for this species as well, however, it was difficult to obtain eggs of the correct stages for two reasons. First, large clutches of eggs were laid at the bottom of a layer of earth approximately every three weeks, assuming the females were not disturbed too often. Since the eggs took approximately three weeks to hatch, and the terrarium was checked for eggs once a week, it was not possible to determine the exact age of a clutch upon collection. The second difficulty was that the developmental time in which neurogenesis took place was only two days out of the three weeks required until hatching. As the chorion of *Archispirostreptus* embryos is so thick and opaque that it is impossible to determine the age of the embryos without overnight fixation in heptane and manual removal of the membrane, most of the embryos fixed were of the wrong stages. Thus, despite the fact that *Archispirostreptus sp.* lays eggs year-round, it is difficult to obtain embryos, which restricted the amount of material available for analysis.

For these reasons, *G. marginata* was used as the principal study organism to examine neurogenesis in millipedes, while the *Archispirostreptus* data proved useful to confirm part of the morphological and molecular results obtained.

3.2. Morphology of the ventral neuroectoderm

3.2.1. Formation of invagination sites

To analyse the development of the ventral neuorectoderm in *G. marginata* I stained embryos at stage 4 with phalloidin-rhodamine, a dye that stains the actin filaments and makes the cell shapes visible. Cell and tissue morphology were then investigated by scanning flat preparations of embryos from apical to basal in the confocal laser-scanning microscope (LSM). At this stage a thickening of the neuroectoderm is already visible (see section 1.4) and the extension of the VNE is clearly demarcated medially by the ventral midline and laterally by the limb buds. I detected dots of high phalloidin-rhodamine staining in apical optical sections of the neuroectoderm of the head segments and the first five leg segments (Fig. 1 D, F, p. 43; Fig 3 A, p. 46). More basal optical sections of the same regions (at a depth of 11 µm to 21 µm from the apical surface of the embryo) revealed that groups of basally enlarged cells are located underneath the strongly stained dots, indicating that these dots are the apical cell processes of the enlarged cells, marking the sites of invagination of neuroectodermal cells (Fig. 3 C, B).

In *Glomeris marginata*, 30 to 32 invaginating cell groups are arranged in a regular pattern of seven rows consisting of four to five invaginaton sites each (Fig. 3 A). Analysis of serial transverse sections revealed that up to eleven cells contribute to an individual invagination site (Fig. 4 A, B, p. 46). Furthermore, the VNE has a multi-layered structure and the invaginating cells of a group do not all occupy a basal position; rather, they can form stacks of cells at different apical-basal positions (Fig. 4 C, D).

A detailed analysis of different embryonic stages revealed that the invagination sites form sequentially in *G. marginata*. The same numbers of invaginating cell groups arise simultaneously in the five head segments and the first two leg segments, while the invagination sites are formed in an anterior to posterior gradient in the remaining

segments (Fig. 1, p. 43). A tight comparison of the relevant embryonic stages showed that the invagination sites are formed in four waves generating 5 to 13 invaginating cell groups each (Fig. 1 G - J; 3 D - G). At stage 2, when the limb buds form, the first invagination sites arise in the medial region of each hemisegment in the five head segments and the first two leg segments (Fig. 1 G; 3 D).

During the second wave of formation of invagination sites at stage 3, new invaginating cell groups arise anterior, posterior and in between the existing invagination sites (Fig. 1 H; 3 E). The next invagination sites to arise form a semicircle around the central region where invagination sites have already formed (Fig. 1 I; 3 F). During the last wave of invagination site generation, invaginating cell groups arise in an anterior-medial region and in between the existing invagination sites (Fig. 3 G). Although the embryo then curves inward and the VNE stretches along the medio-lateral axis, the arrangement in seven rows is maintained (Fig. 1 J).

In summary, the data show that groups of up to eleven invaginating cells are generated in four waves that show a regular pattern in each hemisegment similar to the arrangement of invagination sites in the spider (Stollewerk *et al*., 2001).

3.2.2. Pattern of invagination sites in *Archispirostreptus sp.*

In order to confirm that this mode of neurogenesis is typical for diplopod myriapods, *Archispirostreptus sp.* embryos were stained with phalloidin-rhodamine and the morphology of the VNE was examined. Since this species has not yet been described, nothing is known about the early embryonic development. I divided *Archipirostreptus sp.* development into six stages (Fig. 2, p. 43), which can be compared to the six stages of *G. marginata* development described in section 1.4. (Fig. 1). I found that the chorion can not be removed until approximately 11 days after egg laying. At approximately 12 days after egg laying, a germ band is visible. Comparable to *G. marginata* stage 1 embryos, the first five anterior segments that contribute to the head and the first two leg segments are visible in stage 1.

In *Archispirostreptus sp.* invagination sites begin to form as soon as the germ band is visible in stage 1 embryos (Fig. 2 A, F arrow). By stage 2, an additional leg segment has been formed by the posterior growth zone. At this time in development, almost all invagination sites are present in the head segments and in leg segment 1, and have begun to form in leg segments 2 and 3 (Fig. 2 B, G arrows). Unlike *G. marginata*,

leg segments do not form invagination sites simultaneously and there is a stronger anterior-posterior gradient (Fig 2 B, G arrows). At stage 3 limb buds arise on the antennal, the mandibular and the maxillar segments, as well as on the first two leg segments. Invagination sites are present in the head segments and in leg segments 1 to 4. In the head segments the ectoderm has begun to overgrow the VNE (Fig. 2 C).

This makes it difficult to compare the arrangement of invagination sites to *G. marginata* in detail, since invaginating cell groups are shifted to the center of the hemisegment. The ectoderm overgrows the VNE much earlier in relation to the formation of invagination sites in *Archispirostreptus sp.* than in *G. marginata*. In leg segments one and two, however, the stereotypic arrangement into seven rows of four to five invagination sites can be seen, and the two most lateral invagination sites, which are visible in *G. marginata*, can be identified (Fig. 2 H arrow; compare to 1 F). At this point invagination sites are still visible, and in contrast to *G. marginata*, they continue to be visible when the ectoderm overgrows the VNE (Fig. 2 I, J; Fig. 6, p. 46). In stage 4 a limb bud forms on leg segment 3 and leg segments 3, 4 and 5 form invagination sites (Fig. 2 D). By stage 5 the ectoderm has almost completely overgrown the VNE in the head segments and leg segments 1 - 5 (Fig. 2 E, J arrows).

At stage 5, approximately 13 days after egg laying, a dorso-ventral furrow forms at the level of the postmaxillar segment, so that the embryo curves inward and the head approaches the anal pads at stage 6. At this time in development *Archispirostreptus sp.* embryos have five leg segments and limbs are only visible on leg segments 1 - 3. This is in contrast to *G. marginata*, where the posterior growth zone forms seven leg segments, the first six of which have limb buds, before the embryos curves inwards. By the end of stage 6, the dorsal plates have extended to encompass the yolk completely and the embryo is covered by a waxy cuticle. It hatches after approximately 23 days with three leg pairs and a total of five leg segments.

Thus, in summary, neurogenesis in *Archispirostreptus sp.* follows the same general pattern as in *G. marginata*; however, there is a stronger antero-posterior gradient of invagination site formation. Furthermore, the ectoderm overgrows the VNE before neurogenesis is complete. While neurogenesis is completed in five days in *G. marginata*, it takes two days in *Archispirostreptus sp.*

3.2.3. Invaginating cells express HRP antigen

In order to show that invaginating cell groups really represent neural precursor cells in diplopods, both *G. marginata* and *Archispirostreptus sp.* embryos were stained with the anti-horseradish peroxidase antibody. The HRP antigen has been shown to be present on neuronal cells in myriapods and other arthropods (Jan and Jan, 1982; Stollewerk *et al.*, 2001; Whitington *et al.*, 1991). Double staining of stage 4 embryos of both species with anti-HRP and phalloidin-rhodamine show that the invagination sites are marked strongly by the anti-HRP antibody (Fig. 5, 6, p. 46). This confirms the assumption that invagination sites represent neural precursors.

3.2.4. Proliferating cells are associated with invagination sites

The thickening of the VNE is a result of cell proliferation (Anderson, 1973). To see whether there is a connection between cell proliferation and formation of invagination sites, I double-stained embryos with the mitotic marker anti-phospho-histone 3 and phalloidin-rhodamine to analyse the pattern of cell divisions and to visualize the invagination sites, respectively.

In the VNE of *G. marginata,* mitotic cells are associated with invaginating cell groups and seem to prefigure the regions where invagination sites arise (Fig. 7, p. 48). During formation of the first invagination sites at least one mitotic cell abuts the invaginating cell group (Fig. 7 D - F), while groups of cells and individual cells could be detected in the regions where invagination sites form hours later (Fig. 7 A - C).

In order to confirm these results, live embryos were marked with BrdU, which is incorporated into the DNA when cells divide and acts as a marker of mitosis (Fig. 8, p. 48). Groups of marked cells are visible in the VNE in corresponding regions of each hemisegment, showing that there is a clear pattern of cell division (Fig. 8 A, B arrows). First results from analysis of transverse and sagittal sections indicate that groups of BrdU labeled cells stay together at different apical-basal positions within the VNE (Fig. 8 C).

3.2.5. No decision between epidermal and neural fate

The cells in the VNE of *D. melanogaster* have a choice between an epidermal and a neural fate. It has been shown recently that this decision does not take place in the neurogenic region of the spider. Rather, all cells of the VNE enter the neural pathway (Stollewerk, 2002).

Analyses of transverse and horizontal sections of the VNE of *G. marginata* embryos revealed that the invaginating cell groups detach from the apical surface at stage 6 (Fig. 9, p. 48). At this stage a medial thickening forms in each hemineuromere (Fig. 9 C). Subsequently, the neuroectoderm thickens at the lateral border adjacent to the limb buds (Fig. 9 D) and the whole ventral neuromere sinks into the embryo while the epidermis overgrows the ventral nerve cord (Fig. 9 A, D). At this time, a ladder-like axonal scaffold is already visible on the basal side (Fig. 9 B), suggesting that there is no decision between epidermal and neural fate during the formation of neural precursors in the VNE of *G. marginata*. In *Archispirostreptus* embryos, this process occurs much earlier, before the invagination sites disappear (Fig. 6, p. 46).

Figure Legends 1 and 2

Fig. 1 (A - J) Sequential formation of invagination sites in *G. marginata*. Confocal micrographs of flat preparations of whole embryos (A - E) and the first two leg segments (F - J). Anterior is towards the top, the midline towards the left in F - J. (A, F) No invagination sites are visible at stage 1. (B, G) When the limb buds form at stage 2, the first invagination sites arise in the medial region of each hemisegment (arrow head in G). (C, H) New invagination sites arise anterior, posterior and in between the existing invagination sites (arrow heads in H) during the second wave of neural precursor formation. (D, I) At early stage 4, the next wave generates invagination sites that form a semicircle around the central region where invagination sites have already formed (arrow heads in I). (E, J) At stage 5, the embryo curves inward and the ventral neuroectoderm stretches along the medio-lateral axis. *ant*, antennal segment; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pman*, premandibular segment; *pmax*, postmaxillar segment. Scale bars: 120 µm in A - E; 25 µm in F - J.

Fig. 2 (A - J) Sequential formation of invagination sites in *Archispirosteptus sp*. Confocal micrographs of flat preparations of whole embryos (A - E) and the first two leg segments (F - J). Anterior is towards the top, the midline towards the left in F - J. (A, F) The first invagination sites form at stage 1 (arrow head in F). (B, G) At stage 2, the anterior to posterior gradient of invagination sites is visible (B). More invagination sites are visible in leg segment 1 than in 2 (arrow heads in G). (C, H) All invagination sites are visible in a stereotypic pattern of five rows of 4 - 5 invagination sites in leg segments 1 and 2. The two prominent lateral invagination sites are visible (arrow head in H). (D, I) In stage 4 embryos, the ectoderm has begun to overgrow the VNE (arrow heads in I). Since the embryo has already begun to curve inwards, the invagination sites cannot be seen in all leg segments in D, as the embryos is not completely flat. (E, J) At stage 5, the embryo curves inward and the VNE has been almost completely overgrown by the ectoderm, as marked by the lower arrow head in J. The upper arrow head in J points to axons. *ant*, antennal segment; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pman*, premandibular segment; *pmax*, postmaxillar segment. Scale bars: 125 µm in A - E; 50 µm in F - J.

Figures 1 and 2

Fig. 1

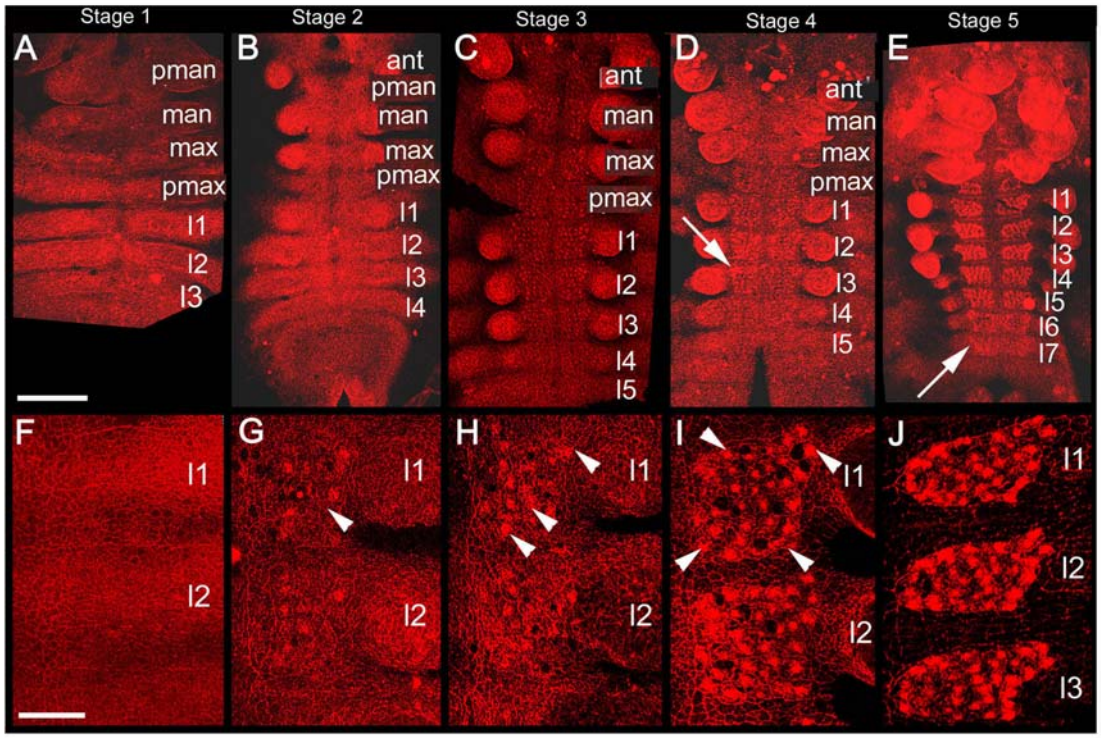


Fig. 2

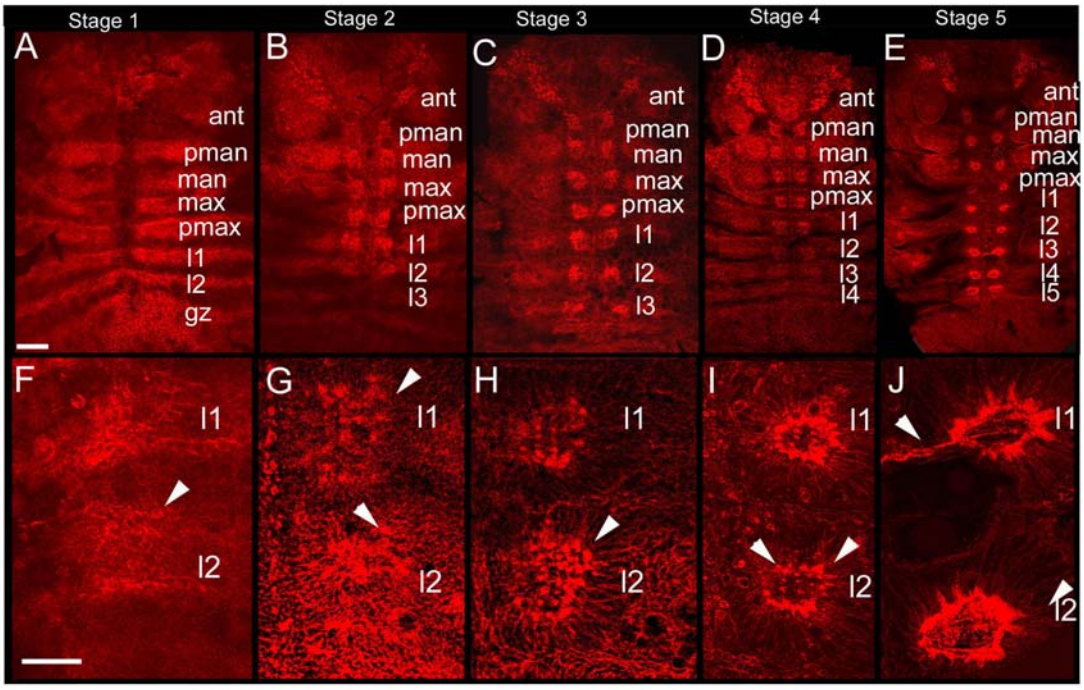


Figure Legends 3 - 6

Fig. 3 Invagination site formation in *G. marginata.* (A) Pattern of invagination sites in the first leg segment of a stage 4 embryo. Anterior is towards the top, the midline towards the left. The invagination sites are arranged in 7 rows consisting of 4 to 5 invagination sites each. The arrows point to two lateral anterior invagination sites that can be easily identified in each hemisegment. (B) Higher magnification of an apical optical section of invagination sites in the ventral neuroectoderm of *G. marginata*. The arrow heads point to two invagination sites. (C) Basal optical section of the same region shown in D. Underneath the dots of high phalloidin-rhodamine staining groups of basally enlarged cells are visible (asterisks). Scale bars: 25 µm in A; 10 µm in B, C. (D - G) Scheme of the four waves of invagination site formation. Anterior is towards the top, the midline towards the left. The first invagination sites to form are marked in red, the second in green, the third in yellow and the fourth in blue.

Fig. 4 (A - D) Apical-basal position of invagination sites in *G. marginata*. Transverse sections of untreated embryos (A, B) and embryos stained for a digoxygenin (DIG) - labeled *GmASH* probe. Basal is towards the top. (A) After formation of the first invagination sites groups of up to 11 cells are visible on the basal side (asterisks) that are attached to the apical surface (arrow). (B) Groups of invaginating cells are located over and above each other (arrow heads) after formation of the third wave of neural precursors (asterisks and crosses). The cells are still attached to the apical surface (arrow). (C, D) *GmASH* is transiently expressed in the invaginating cell groups which are located at different apical-basal positions (arrows). Some of the invaginating cell groups form stacks (arrow head). Scale bar: 10 µm in A - D.

Fig. 5 (A - C) Invagination sites express the neural marker HRP. *G. marginata* stage 4 embryos double stained with phalloidin-rhodamine (red) and anti-HRP antibodies (green). Anterior is towards the top and the midline towards the left. The overlay of A and B is shown in C. *l1* and *l2*, leg segments 1 and 2. Scale bar: 25 µm in A - C.

Fig. 6 (A, B) Invagination sites express the neural marker HRP. *Archispirostreptus sp.* stage 4 embryos double stained with phalloidin-rhodamine (red) and anti-HRP antibodies (black). Anterior is towards the top and the midline towards the left, leg segment 1 is shown here. The ectoderm has already begun to overgrow the VNE, as shown by the arrows. Scale bar: 50 μm in A, B.

Figures 3 - 6

Fig. 3

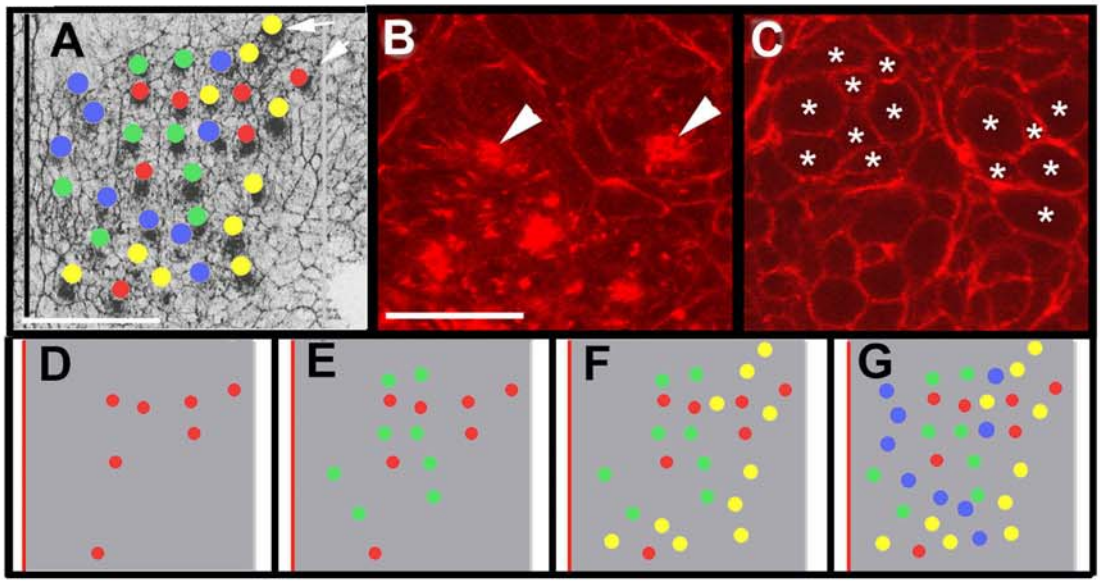


Fig. 4

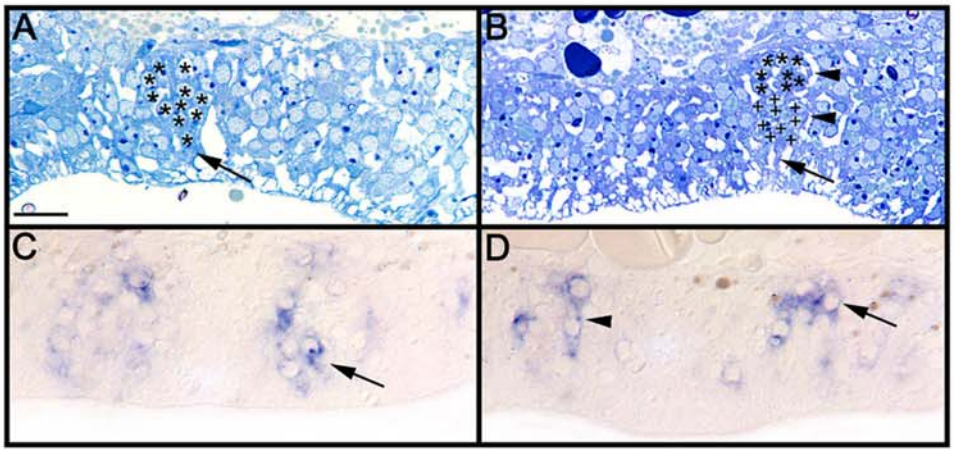


Fig. 5

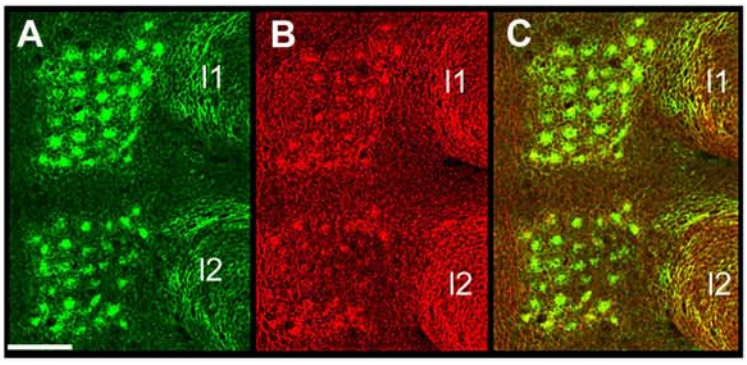


Fig. 6

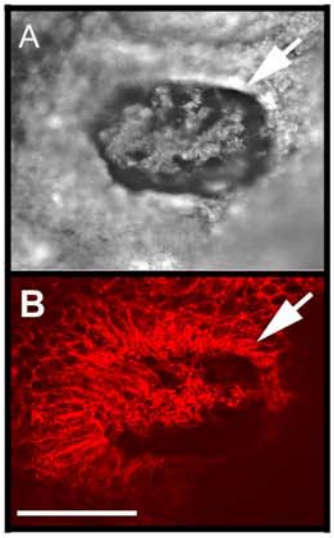


Figure Legends 7 - 9

Fig. 7 (A - F) Proliferating cells are associated with invagination sites. Confocal micrographs of flat preparations of embryos stained with phalloidin-rhodamine (red) and anti-phospho histone 3 antibodies (green). Anterior is towards the top and the midline towards the left in D - F. (A - C) Single mitotic cells are associated with invagination sites that have already formed. The arrow in A points to an invagination site. The arrow in B points to a mitotic cell located at the same position as the invagination site indicated in A. The overlay of A and B is shown in C. (D - F) During formation of the first invagination sites in the central region of the hemisegment (arrow heads and arrow in D), mitotic cells are located at the centre of the invagination sites (arrow heads in E and F) or close to the invagination site (arrows in E and F). *l1* to *l2*, leg segments 1 to 2. Scale bars: 50 µm in A - C; 10 µm in D - F.

Fig. 8 (A - C) BrdU labelling of mitotically active cells. (A, B) Anterior is towards the top. A clear pattern of cell division can be seen in the VNE of stage 4 G. marginata embryos in positions where the last invagination sites form (arrows in B; compare to 3 G). *l1* to *l3*, leg segments 1 to 3 (C) A dorso-ventral sagittal section of one hemisegment of a BrdU labeled embryo. Anterior is to the left, dorsal to the top. The lines mark the borders of the hemisegment. Groups of labeled cells are visible in different dorso-ventral positions. Scale bars: 120 µm in A, 25 µm in B, 10 µm in C.

Fig. 9 (A - D) The epidermis overgrows the ventral nerve cord after formation of the neuropil. Flat preparations (A, B) and transverse semi-thin sections of the ventral nerve cords at stage 6. (A) Apical optical section of the ventral nerve cord of an embryo stained with phalloidin-rhodamine. The ventral neuromeres sink into the embryo (asterisk) and the epidermis overgrows the nerve cord (arrow). (B) Basal optical section of the same region shown in A. A ladder-like neuropil has been formed by the invaginated cells (arrow). (C) Transverse section of the hemineuromere of leg segments 1 at early stage 6. At this stage a medial thickening forms (arrow head). The asterisk indicates the midline. (D) At late stage 6 an additional thickening has formed at the lateral border of the hemineuromere and the central part sinks into the embryo. The asterisk indicates the midline. *l1* to *l3*, leg segments 1 to 3. Scale bar: 10 µm in A - D.

Figures 7 - 9

Fig. 7

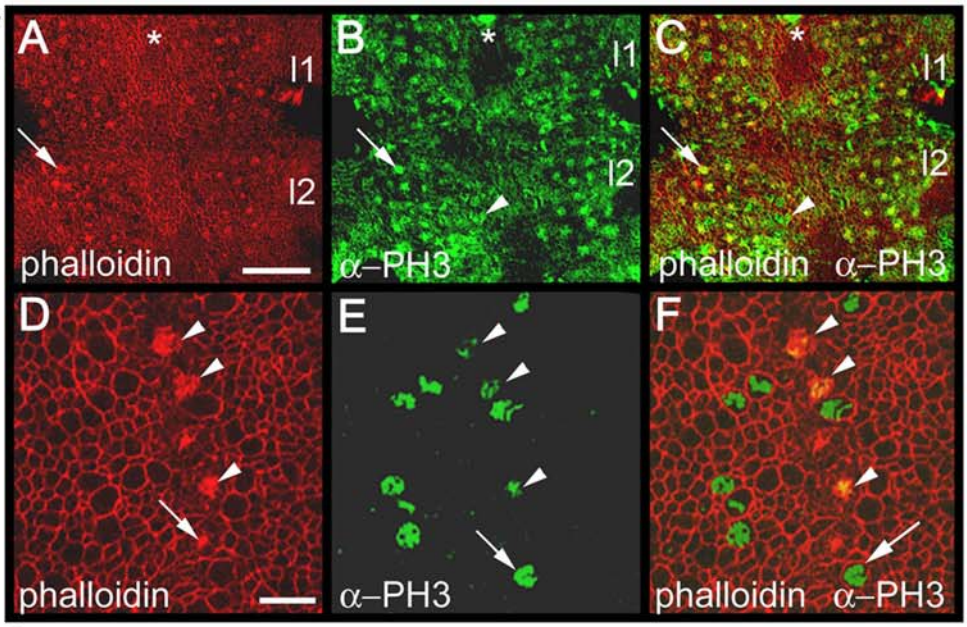


Fig. 8

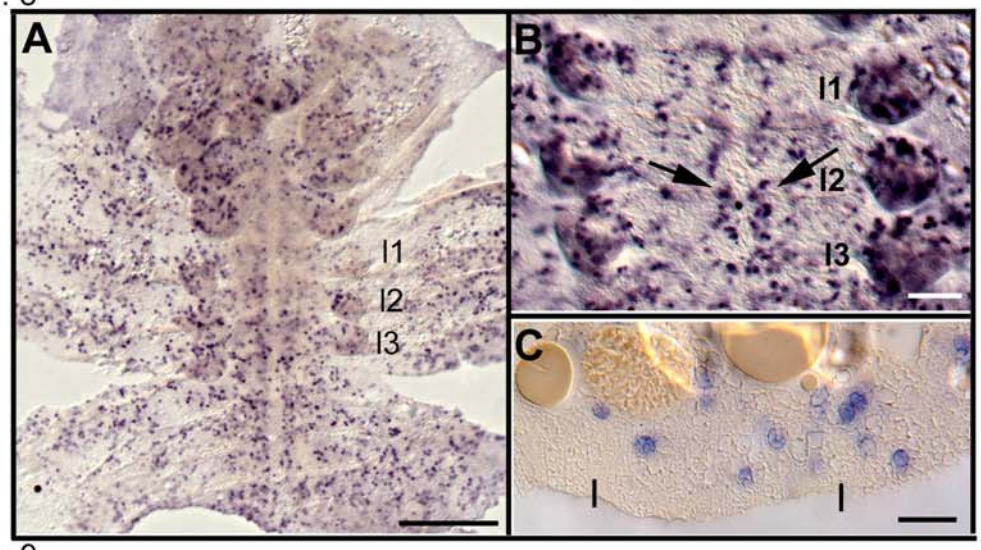
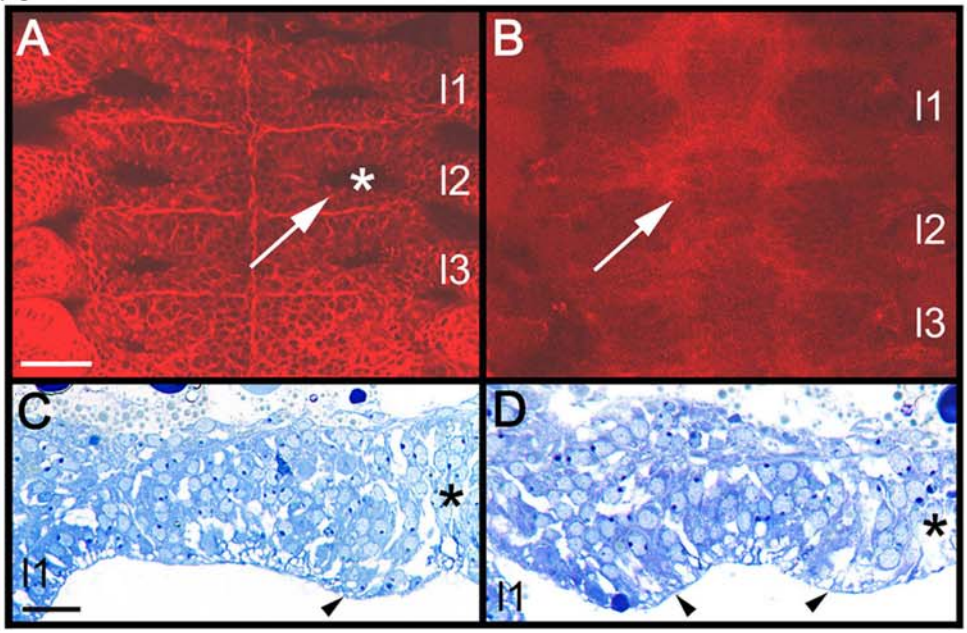


Fig. 9



3.3. Molecular mechanisms of nervous system development

The sequential formation and the regular pattern of the invagination sites in the VNE of *G. marginata* suggest that, similar to *D. melanogaster* and the spider *C. salei*, proneural and neurogenic genes regulate the recruitment of neural precursor cells from the neurogenic region. To see whether there is an evolutionarily conserved mechanism for patterning the VNE, and whether the expression of molecular markers of cell specification is comparable between *C. salei* and *G. marginata,* homologs of the corresponding *D. melanogaster* genes were isolated.

Genes that were isolated for this work were divided into four groups: genes involved in early neurogenesis, neurogenic genes, VNE patterning genes and markers of cell fate specification. Conserved features of the genes of interest allowed for the design of degenerate primers (see section 2.4.2.4.) and led to the amplification of small fragments. In most cases it was necessary to obtain larger fragments by rapid amplification of cDNA ends in order to study the expression of the gene. THe obtained sequences are listed in the Appendix, section 6.1.

3.3.1. Genes involved in early neurogenesis

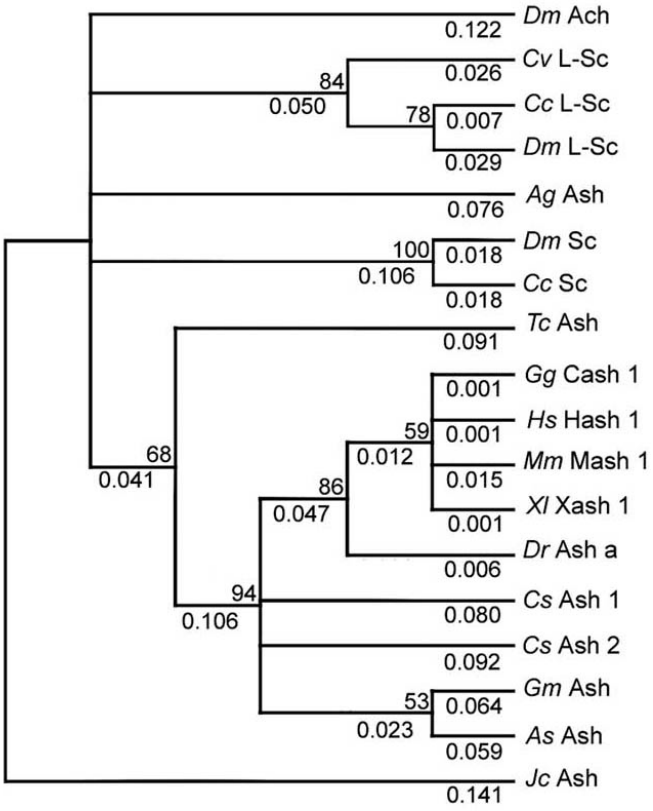
The genes I was interested in cloning to study the molecular mechanisms of early neurogenesis in myriapods were *ASH* genes, *pannier, atonal* and *daughterless* homologs and members of the group B of the *Sox* family. Cloning homologs of bHLH genes was difficult because the bHLH domain, while highly conserved, is very short and has a high percentage of degenerate codons, making primer design difficult and leading to the amplification of many unspecific products. Conservation outside of this domain is very low. A similar problem arose when attempting to clone *Sox* homologs, which are also only conserved in the HMG box and very similar to each other in this region. For this reason, multiple original fragments were identified and larger fragments of the most frequent fragment were obtained via rapid amplification of cDNA ends (RACE).

3.3.1.1. *Achaete scute* homologs

RACE of the 5´and 3´ ends of *GmASH* resulted in a 1017 bp fragment with a 837 bp open reading frame (ORF), *Archispirostreptus* 5´ and 3´ RACE led to a 1320 bp fragment with an open reading frame of 864 bp. Both sequences have a single start codon with a short conserved motif also found in the *CsASH* genes, as well as upstream and downstream stop codons. Comparision of the *GmASH* and *AsASH* deduced amino acid sequences led to an overall similarity of 61%, with 86% identical amino acids in the bHLH domains. The *GmASH* deduced amino acid sequence is 83% identical to *Homo sapiens* achaete-scute complex homolog-like 1, while the *Archispirostreptus* sequence is 81% identical to the *Gallus gallus* transcriptional regulator *CASH*, but both of these similarities only cover the bHLH domain. Outside of this domain, it is only possible to align the start codons and a short conserved domain at the end of the protein, even between closely related species. The alignment of the bHLH domains with other ASH proteins showed that, in contrast to insects, diplopod myriapod sequences, like their spider and vertebrate homologs, have a reduced loop (alignment 1). A tree was constructed from an alignment of the bHLH domains of nine insect, five vertebrate, two *Cupiennius salei* and the myriapod sequences (Tree 1). The node joining the myriapod, spider and vertebrate sequences has very high bootstrap support (94), while that joining the insects has low support.

Alignment 1. Comparison of the basic domain, the two helices and the loop region of *GmASH* with the bHLH region of another diplopod, two insect, two vertebrate and two spider sequences. Note that the loop region of *G. marginata* ASH is reduced to the same extent as in the spider and vertebrate bHLH domains. The *GmASH* bHLH domain shows the highest similarity to the same region of the millipede *Archispirostreptus spec*. See text for further details. Species: *Ag*, *Anopheles gambiae*; *As*, *Archispirostreptus spec*.; *Cs*, *Cupiennius salei*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus.*

	basic	helix	loop	helix
Dm Ac	RRNARERNRVKQVNNGFSQLRQHIPAAVIADLSNGRRGIGPG-ANKKLSKVSTLKMAVEYIRRL
Tc ASH	.K.....R.....AN.....NFIA.AFESNS.----..G.....E..R.....S.
Gm ASH	...E.....L..M..AT..E.V.N---------------.VK...M...E..RS..D..K..
As ASH	...E..S....L..L..AT..E.V.N---------------.SK...M...E..RS.....K.
Cs ASH1	...E.....R...L..AT....V.N----------------.RS..M...E..RS..Q...Q.
Cs ASH2	...E.....RL..L..AN....V.N---------------SSK...M...D..RS.....KQ.
Hs ASH1	...E.....L..L..AT..E.V.N---------------.A....M...E..RS.....A.
Mm ASH1	...E.....L..L..AT..E.V.N---------------.A....M...E..RS..Q...A.



Tree 1. Phylogeny of the conserved domain of the *achaete-scute* homologs. The tree was constructed from an alignment of the bHLH domains of nine insect, five vertebrate, two spider and two myriapod sequences using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. Both myriapod homologs group outside the insect genes together with the spider and vertebrate homologs. Genes: L-sc, Lethal of scute; Sc, Scute; Ac, Achaete; Ash, Achaete-Scute Homolog; Species: *Ag*, *Anopheles gambiae*; *As*, *Archispirostreptus spec.*; *Cc*, *Ceratitis capitata*; *Cs*, *Cupiennius salei*; *Cv*, *Calliphora vicina*; *Dm*, *D. melanogaster*; *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Jc*, *Junonia coenia*; *Lc*, *Lucilia cuprina*; *Mm*, *Mus musculus*. *Tc*, *Tribolium castaneum*; *Xl*, *Xenopus laevis*.

3.3.1.2. Expression pattern of *GmASH*

GmASH transcripts were first detected before formation of the limb buds at stage 1. At this time no invagination sites are visible in the VNE (Fig. 10 A, p. 56). The gene is expressed in neuroectodermal cells in the middle of each hemisegment in the head and the first two leg segments at heterogenous levels (Fig. 10 F). Groups of cells express high levels of the gene, while there is a weak uniform expression in the remaining regions (Fig. 10 F). At stage 2, invagination sites arise in the expression domains of *GmASH* (Fig. 10 B). At this time transcripts can be detected anterior, posterior and in between the first invagination sites (Fig. 10 G; Fig. 11 A, p. 56). Again the next invagination sites to arise are generated in the regions of *GmASH* expression (Fig. 10 C). Although the gene is simultaneously expressed in the head segments and the first two leg segments, the expression domains in the antennal, premandibular, mandibular and maxillar segments seem to be smaller than in the remaining segments (Fig. 11 A - D).

At stage 3 the expression domains of *GmASH* form a semicircle around the area where invagination sites have already formed (Fig. 10 H; Fig. 11 B). This expression

pattern again prefigures the regions where invagination sites will be formed hours later (Fig. 10 D). Before the last wave of formation of invagination sites *GmASH* is expressed in the corresponding regions in between and anterior-medial to the existing invagination sites. In addition, the gene is transiently expressed in the invaginating cell groups and in the neural precursors of the PNS in groups of cells in the head and leg appendages and the dorsal plates (Fig. 10 J; Fig. 11 C, D).

In summary, the data show that the *G. marginata achaete-scute* homolog is expressed prior to invagination of the neural precursors in the appropriate regions, similar to the spider gene (Stollewerk *et al*., 2001).

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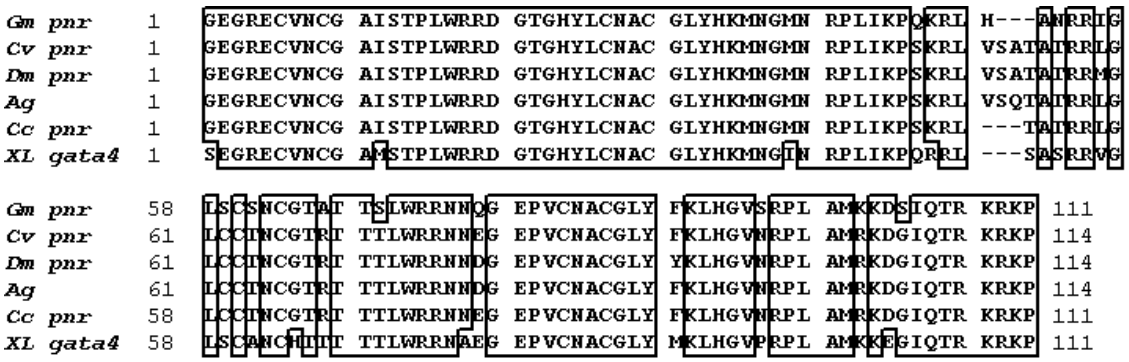
3.3.1.3. *G. marginata pannier* homolog

In the case of *pannier*, nine positive clones were identified which fell into two groups, one with six and the other with three sequences. BOth primary sequences are shown in section 6.1. RACE primers were designed based on the six original clones and both 3' and 5' RACE yielded a single 1297 nucleotide with a putative amino acid sequence of 435. No upstream or downstream stop codons could be identified. This sequence is 69% identical to the *Ceratitis capitata* pannier protein. An alignment of the GATA zinc finger domains shows that this region is highly conserved with only 23/115 informative positions (Alignment 3). The *G. marginata* sequence is in between the insect sequences and the vertebrate GATA4 sequences.



Alignment 3 Comparision of the highly conserved GATA zinc finger of GmPannier to this domain from four insects and one vertebrate sequence. Since this domain of the vertebrate GATA4 proteins is virtually identical, only one was included. Identical amino acids are outlined. Sequences: pnr; pannier; the *Ag* sequence is not yet annotated. Species: *Ag*, *Anopheles gambiae*; *Cc*, *Ceratitis capitata*; *Cv*, *Calliphora vicina*; *Dm, D. melanogaster; Gm, G. marginata; Xl, Xenopus laevis*

3.3.1.4. *Pannier* expression

G. marginata pannier expression is first visible in stage 3 embryos in cells at the outer edge of the dorsal plates which demarcate the limit between the germ band and the dorsal extra-embryonic membrane (Fig. 12 A, B and arrows, p. 56). This expression becomes stronger in stages 4 (Fig. 12 C, D and arrows). In stage 5, expression is also present at the borders between the dorsal plates (Fig. 12 E, arrow). At stage 6, when dorsal closure begins, *pannier* expressing cells are visible between dorsal plates and as a thin line of cells demarcating the most dorsal edge of the embryo from anterior to posterior (Fig. 12 F - H, arrows).

Figure Legends 10 - 12

Fig. 10 (A - J) *GmASH* prefigures the regions where invagination sites arise. Confocal micrographs (A - E) and light micrographs of (F - J) of the first leg hemisegment. Anterior is towards the top, the midline is towards the left. (A, F) *GmASH* is expressed at heterogenous levels in the central region of the hemisegment before formation of the invagination sites at stage 1 (arrow heads). (B, G) The first invagination sites (arrow heads in B) arise in the expression domains of *GmASH* at stage 2. At this time the gene is expressed in distinct regions of the hemisegment (arrow heads in G). In addition, *GmASH* shows transient expression in the invaginating cells. (C, H) At stage 3, the second wave generates invagination sites in the regions prefigured by *GmASH* (arrow heads in C, compare to G). At this time, the expression domains of *GmASH* form a semicircle around the central region where invagination site have already formed (arrow heads in H). (D, I) Again invagination sites arise in the expression domains of *GmASH* (arrow heads). At this time the gene is re-expressed in distinct regions (arrow heads in I). (E, J). In these regions the last invagination sites arise during the fourth wave of neural precursor formation at late stage 4. *GmASH* expression is transiently maintained in the invaginating cell groups (arrow head in J). Scale bar: 25 µm in A - J.

Fig. 11 (A-D) Expression pattern of *GmASH*. Flat preparations of whole embryos stained for a DIG-labeled *GmASH* probe. Anterior is towards the top. (A) An anterior to posterior gradient of *GmASH* expression is visible in the neurogenic regions of the embryo. The medio-lateral extension of the *GmASH* expression domain is smaller in the head segments. An identical *GmASH* expression pattern is visible in the leg segments 1 and 2 (arrow), while the former expression pattern of anterior segments can be detected in leg segment 3 (arrow; compare to Fig. 7 F, G). (B) At stage 3, *GmASH* expression forms a semicircle around the central region of the hemisegments (arrow). The former expression pattern of the anterior segments is now visible in leg segment 3 (arrow). (C) *GmASH* expression has extended posteriorly to leg segment 5. The arrow points to the transient expression of *GmASH* in the invaginating cell groups. (D) After formation of all invagination sites, *GmASH* is still expressed in about half of them. In addition, the gene is expressed in the precursors of the PNS (arrows). *ant*, antennal segment; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pman*, premandibular segment; *pmax*, postmaxillar segment. Scale bar: 120 µm in A - D.

Fig. 12 (A - H) Expression pattern of *G. marginata pannier*. (A - D) Flat preparations of whole embryos stained for a DIG-labeled *pannier* probe. Anterior is towards the top. *l1* to *l3*, leg segments 1 to 3. (A, B) Stage 3 embryos, *pannier* expression is visible in cells at the outer edge of the dorsal plates which demarcate the limit between the germ band and the dorsal extra-embryonic membrane, shown by arrows. (C, D) In stage 4 embryos, this expression is stronger as shown by arrows. (E - H) Whole mount pictures of embryos stained for a DIG-labeled *pannier* probe. The embryos are curved inward and the head approaches the anal pads. (E) Side view of a late stage 5 embryo. The head is in the upper right, the anal pads in the lower right. *Pannier* is expressed between the dorsal plates, as shown by the arrow. (F - H) Stage 6 embryo. (F) Side view, the head is in the upper right, the anal pads in the lower right. *Pannier* expressing cells are visible as a thin line of cells demarcating the most dorsal edge of the embryo from anterior to posterior, as shown by the arrow. (G) Dorsal view , *pannier* is expressed in a thin line of cells at the most dorsal edge of the embryo on both sides before dorsal closure, as shown by the arrows. (H) Ventral view of the embryo. The line of *pannier* expressing cells is visible on the dorsal side of the head as shown by the arrows. Anterior is to the top. Scale bar: 120 µm in A, C; 50 µm in B, D.

Figures 10 - 12

Fig. 10

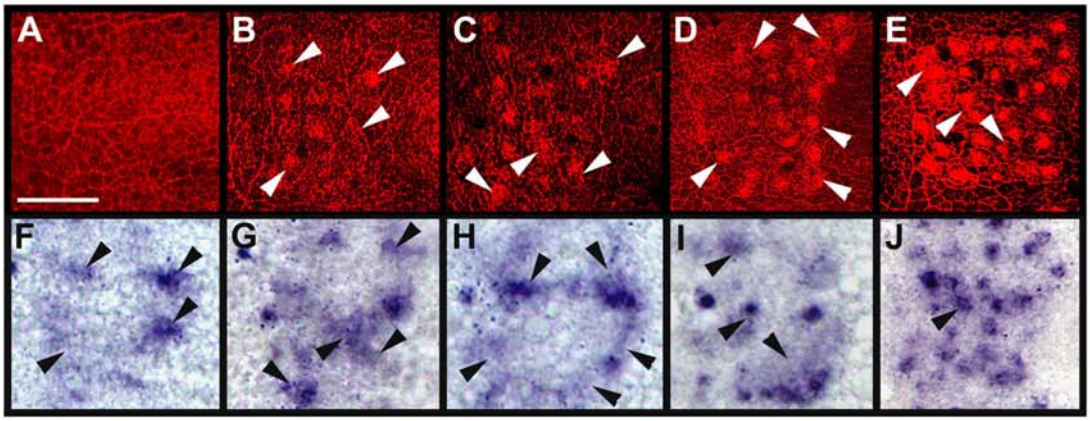


Fig. 11

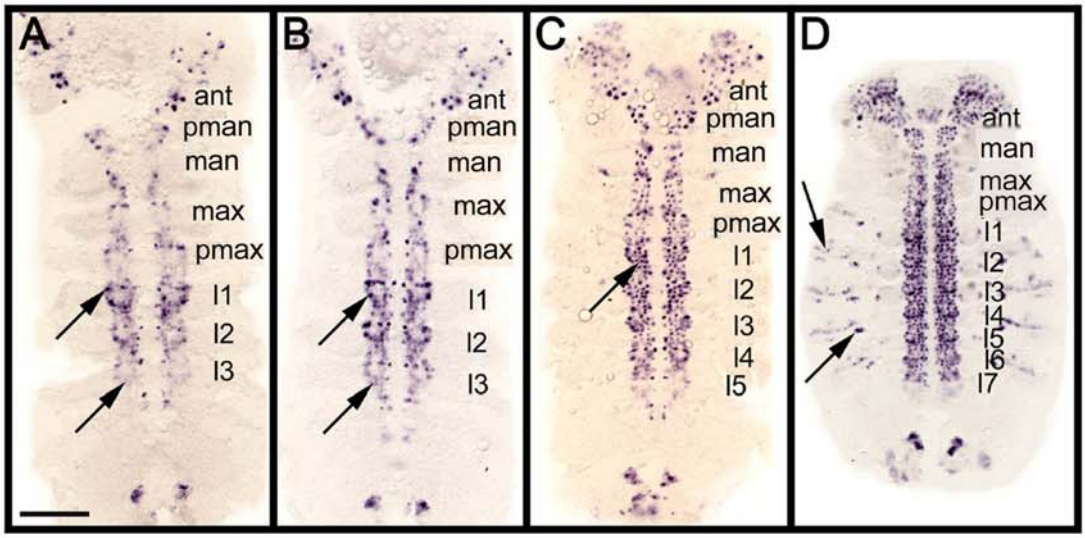
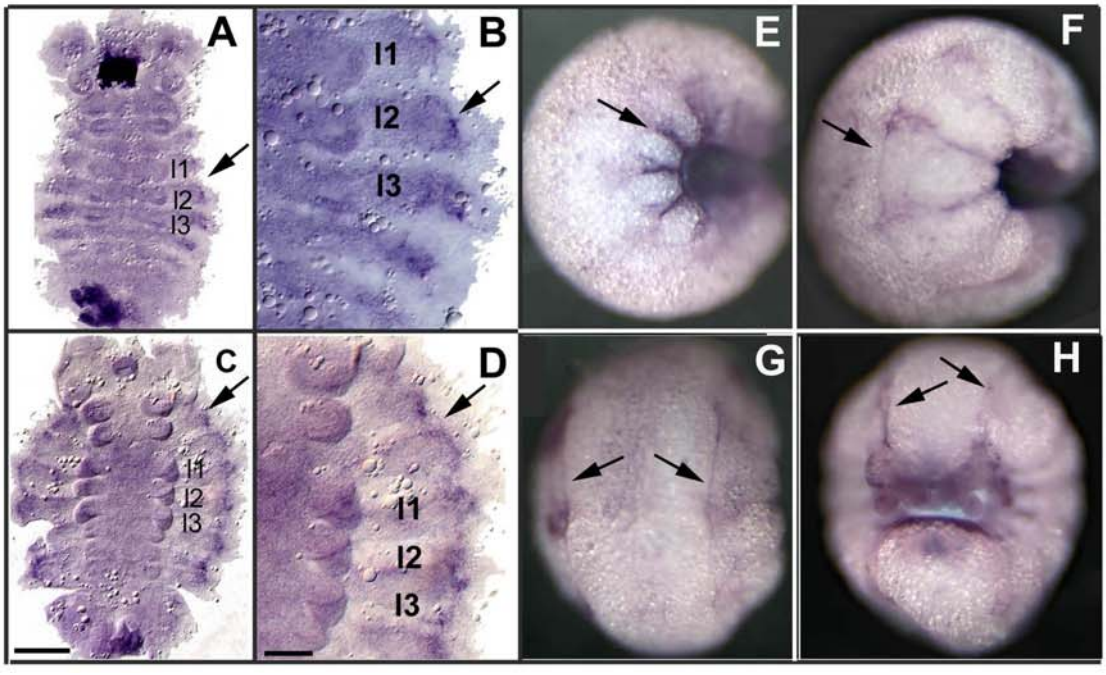


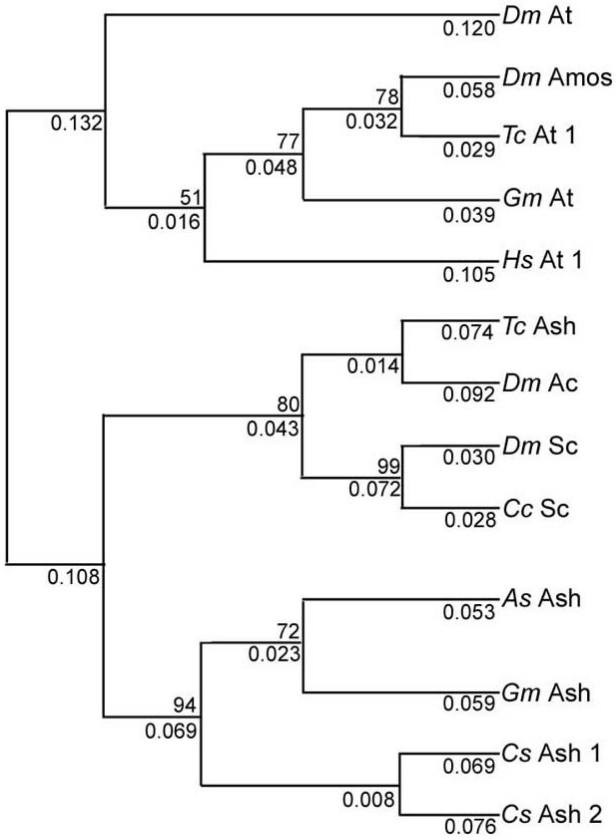
Fig. 12



3.3.1.5. *Atonal* homologs

Twelve positive clones were identified, of which three were different from the rest on a nucleotide level. On the amino acid level all primary clones were identical and showed an *atonal* type bHLH domain. Three prime RACE primers were designed based on the most predominant sequence, and a 1301 base pair *G. marginata atonal* (*GmAt*) fragment was isolated. The end of the *atonal* bHLH domain in *G. marginata*, as in insects, also represents the end of the coding sequence, and so in the +1 reading frame, only the first 123 nucleotides are coding, ended by a stop codon and the rest is three prime UTR (see section 6.1). Nevertheless, antisense RNA probes from this fragment yield a specific expression pattern.

The deduced amino acid sequence of the bHLH domain shows 80% similarity to the *D. melanogaster* Amos protein, 79% to the *Gallus gallus* Atonal homolog CATH1 and 70% to *D. melanogaster* Atonal. However, a tree of the Atonal proteins from *G. marginata, Tribolium castaneum, Homo sapiens* and *D. melanogaster* with *D. melanogaster* Amos and a subset of Achaete Scute homologs shows that *D. melanogaster* Amos groups with the *T. castaneum* and *G. marginata* sequences with relatively high bootstrap support (77%), while *D. melanogaster* Atonal appears relatively derived, since it does not cluster with the other Atonal proteins (Tree 2). There is, however, a clear split between the Achaete-Scute proteins and the Atonal type bHLH domains (80% for the insects; 94% for the spider and millipede sequences), which again confirms that *GmAtonal* is a real Atonal homolog.



Tree 2. Phylogeny of the conserved domain of Atonal and Achaete-scute type proteins. The tree was constructed from an alignment of the bHLH domains of seven insect, one vertebrate, two spider and three myriapod sequences using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. There is a clear grouping of the Achaete-Scute proteins together, and *G. marginata* Atonal groups with the other Atonal proteins. Genes: At, Atonal; Ac, Achaete; Ash, Achaete-Scute Homolog; Sc, Scute Species: *As*, *Archispirostreptus spec.*; *Cc*, *Ceratitis capitata*; *Cs*, *Cupiennius salei*; *Dm*, *D. melanogaster*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Tc*, *Tribolium castaneum*.

3.3.1.6. Expression pattern of *GmAt*

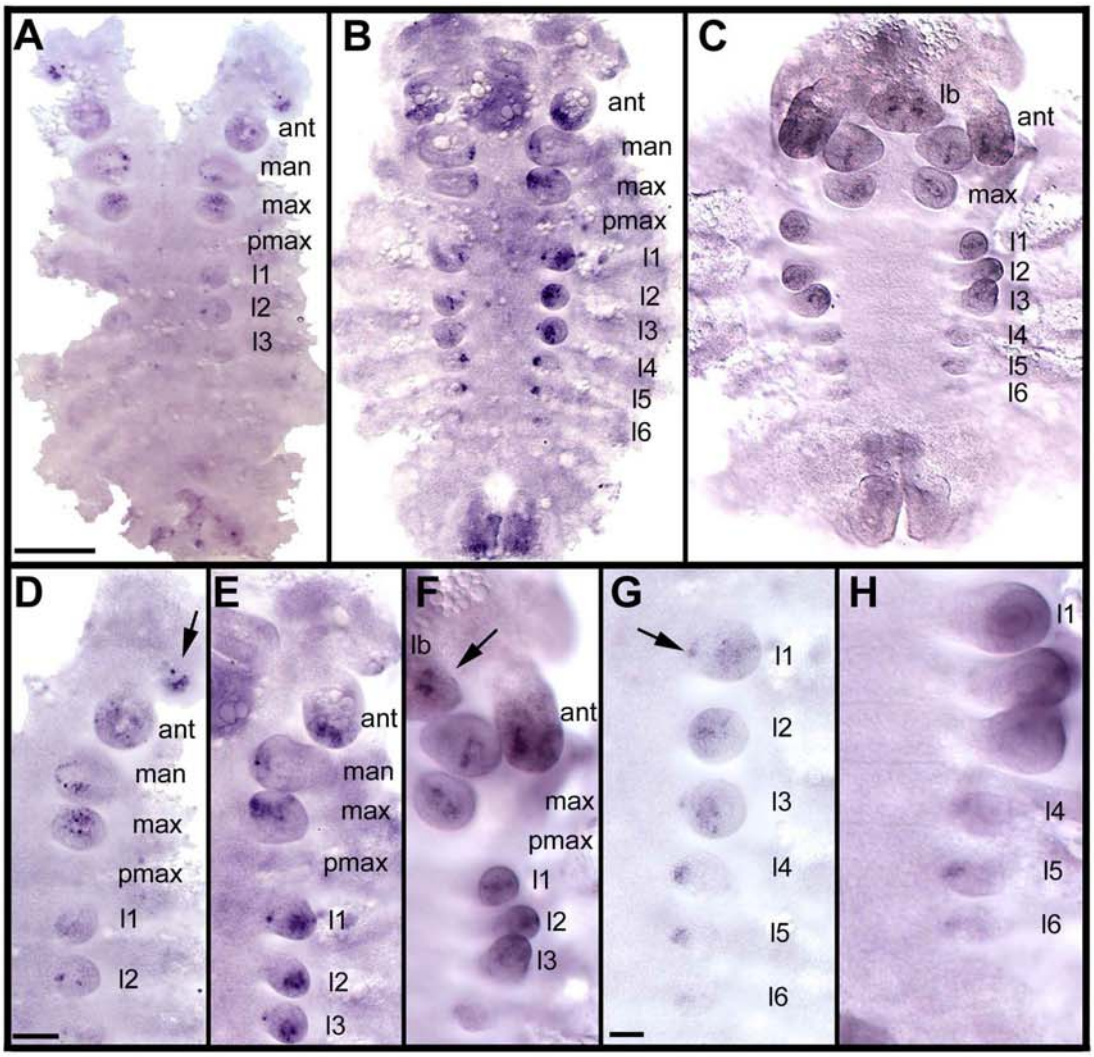
GmAt is expressed exclusively in precursors of the PNS (Fig. 13, p. 60). Transcripts were first detected after the formation of the head appendages at stage 3 in groups of cells at the tip of the antenna, mandible and maxilla (Fig. 13 A, D). In addition at this stage transient expression was observed in a group of cells on the most lateral part of the head, where the oecelli will probably arise (Fig. 13 D arrow). In leg segments 1, 2, and 3, several groups of cells express *atonal* in the appendages while no expression could be observed in the post-maxillae (Fig. 13 A). Stage 4 embryos express *atonal* in groups of cells at the tips of the antennae, the mandibles and the maxillae as well as in leg buds of segments 1, 2 and 3 (Fig. 13 B, E). In addition, expression is now present at the base of the appendages in leg segments 1 to 6 (Fig. 13 E, G). Atonal continues to be expressed in all appendages at stage 5, and additional staining is visible in four cell groups at the tip of the labrum, which may correlate with the position where four sensory bristles will form (Dohle, 1964) (Fig. 13 C, F arrow, H). In summary, *GmAt*, like its *D. melanogaster* counterpart, is expressed in regions where sensory organs are most likely to develop and there is no expression in the CNS (Jarman *et al.*, 1996).

Figure Legend 13

Fig. 13 (A - H) Expression pattern of *G.marginata atonal* (*GmAt)*. Flat preparations of whole embryos stained for a DIG-labeled *GmAt* probe. Anterior is towards the top. (A, D) At stage 3, *GmAt* expression is visible in in groups of cells in the antennea, mandible and maxilla, as well as in legs 1 and 2. The arrow in D points to the transient expression of *GmAt* in a group of cells in the most lateral part of the head. (B, E, G) By stage 4, *GmAt* is expressed at the tips of the head appendages and legs 1 - 3. Additional expression is visible at the base of the appendages in leg segments 1 - 6, see arrow in G. (C, F, H) Stage 5 embryos express GmAt in all appendages and at the tip of the labrum, as is shown by the arrow in F. *ant*, antennal segment; *l1* to *l7*, leg segments 1 to 7; *man,* mandibular segment; *max*, maxillar segment; *pmax,* postmaxillar segment. Scale bars: 120 µm A - C; 50 µm in D - F; 25 µm in G, H.

Figure 13

Fig. 13



3.3.1.7. *Daughterless* homolog

Despite the very derived bHLH domain of *D. melanogaster daughterless*, the only homolog to the mammalian E-box proteins, it was possible to isolate 12 primary clones, of which 10 were identical. RACE PCR was only performed on the most highly represented fragment (section 6.1 for original fragments). Five prime RACE yielded a 1711 bp fragment with an open reading frame leading to a putative amino acid sequence of 569 aa. The beginning of the gene is conserved and can be aligned with the *D. melanogaster* homolog as can one short domain and the bHLH domain, but the other parts cannot be reliably aligned, leading to an overall similarity of 26% to *D. melanogaster daughterless* with to 84% identities in the short bHLH domain.

3.3.1.8. Expression pattern of *G. marginata daughterless*

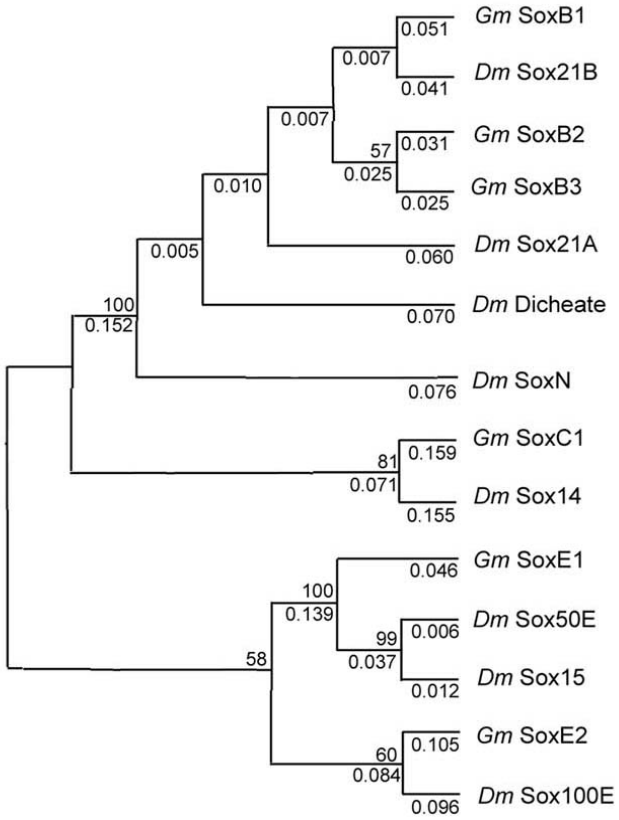
Daughterless is expressed ubiquitously in *G. marginata* embryos, beginning at stage 2 (Fig. 14, p. 65). At this point in development, expression is homogenous (Fig. 14 A) while later, around stage 3 (Fig. 14 B, D, E, F arrow), there is a heterogeneity in expression levels. Stronger expression is visible in the head and in the VNE on either side of the midline, while expression levels in the ventral midline itself are relatively low (see asterisks in Fig. 14 C, E, F). In the VNE, heterogenous expression levels are visible in the segments where invagination sites have already formed, in stage 3 (Fig. 14 B, D, E, F) in leg segments 1 - 4 and by stage 5 (Fig. 14 C) in leg segments 1 - 6. Thus, while *G. marginata daughterless* is expressed in the whole embryo, as is the *Drosophila* homolog, in contrast to *Drosophila*, levels of expression are not homogenous throughout the embryo.

3.3.1.9. *Sox* homologs

Due to the high conservation of the HMG box, 19 independent clones were identified, of which 10 had the highest homology to the *Gallus gallus* Sox 2 homolog (*GmSoxB1*), three were most similar to the *Xenopus laevis* Sox 11 protein (*GmSoxB2*), three were closest to the vertebrate Sox 21 proteins (*GmSoxB3*), two showed the highest similarity to the *D. melanogaster* Sox 14 amino acid sequence (*GmSoxC1*) and two different clones were most similar to the vertebrate Sox 8 proteins (*GmSoxE1, GmSoxE2*). These fragments are aligned in section 6.1.

A phylogenetic comparision of the six original *G. marginata* Sox deduced protein sequences to the corresponding regions of the *D. melanogaster* Sox sequences available in the database showed that the members of the Sox family found in *D. melanogaster* and those I identified in *G. marginata* are, for the most part, not lineage specific duplications. The high bootstrap support for the division into groups B, C and E (respectively 100%; 87% and 58%), together with the fact that most of the *G. marginata sox* genes isolated have the highest similarity to vertebrate homologs, indicate that these groupings are phylogenetically old (Tree 3).

RACE primers were designed for the 3' end of the most highly represented fragment (S*oxB1*) and yielded a 1566 bp fragment with an open reading frame from 1 - 882, encompassing the HMG domain, two conserved amino acids at the 3' end (HM) and a stop codon followed by the 3' UTR. The complete fragment shows the highest similarity to the hemichordate *Saccoglossus kowalevskii Sox1/2/3* homolog (43%, 100% in the HMG domain), but similarities are restricted to the HMG domain. Alignement with other Sox proteins shows that the putative amino acid sequence of *G. marginata marginata SoxB1* places it in group B with higher similarity to the vertebrate *Sox 2* genes than to *D. melanogaster SoxNeuro* or *Dichaete*.



Tree 3. Phylogeny of the conserved domain of members of the *D. melanogaster* and *G. marginata* Sox family. The tree was constructed from an alignment of the High Mobility Group (HMG) domains of eight *D. melanogaster* (*Dm*) and six *G. marginata* (*Gm*) sequences using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. There is a clear division into three family groups: Group B with three *G. marginata* and four *D. melanogaster* members , Group C with one sequence per species, and Group E with three *D. melanogaster* and two *G. marginata* sequences. Each group has relatively high bootstrap support.

3.3.1.10. Expression pattern of *GmSoxB1*

GmSoxB1 is visible as early as developmental stage 1 and shows very strong expression by stage 2 in the complete VNE where invagination sites will form and and in regions where the brain will develop as well as at the tip of the antennal, mandibular and maxillar appendages (Fig. 15 A, arrow, p. 65). This expression persists throughout invagination site formation and is visible in each newly formed segment (Fig. 15 B, C) as well as in the tip of the labrum when this is formed (Fig. 15 B, C and arrows). No expression of *GmSoxB1* is visible in the midline (demarcated by an asterisk in Fig. 15 A and B), in the leg appendages or in the PNS of the rump. *GmSoxB1* is thus expressed in the VNE before invagination sites are visible morphologically (compare to Fig. 5) and at the tips of the head appendages.

Figure Legends 14 and 15

Fig. 14 (A - F) Expression pattern of *G. marginata daughterless*. Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A) At stage 2 expression is homogenous. (B, D - F) Stage 3 embryos have heterogenous expression in the VNE of leg segments 1 - 3, see arrow in F. (C) By stage 5, expression is heterogenous in the complete VNE. *ant*, antennal segment; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pmax*, postmaxillar segment. Scale bar: 120 µm in A - C, 25 µm in D - F.

Fig. 15 (A - C) Expression pattern of *G. marginata SoxB1*. Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A) Stage 2 embryos. Expression throughout the VNE before invagination site formation and at the tips of the head appendages, as shown by the arrow. No expression is visible in the midline, which is marked by asterisks. (B) In stage 3 embryos three additional leg segments have been formed by the posterior growth zone and express *GmSoxB1* in the VNE and at the tips of the head appendages, as shown by the arrow. The asterisk marks the ventral midline, where there is no expression. (C) Strong expression in all segments of the VNE continues, as does the expression at the tips of the head appendages (arrow) and at the tip of the labrum, which has been formed as shown by the arrow head. *ant,* antennal segment; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pmax*, postmaxillar segment. Scale bar: 120 µm in A - C.

Figures 14 and 15

Fig. 14

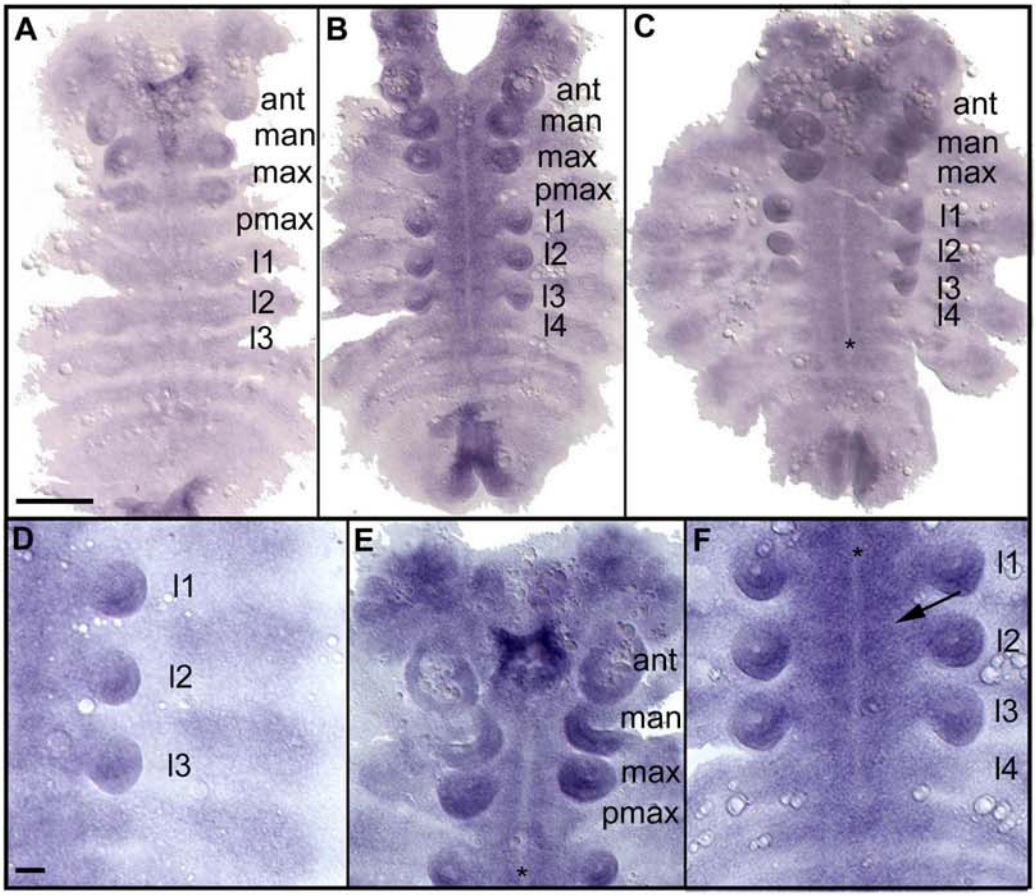
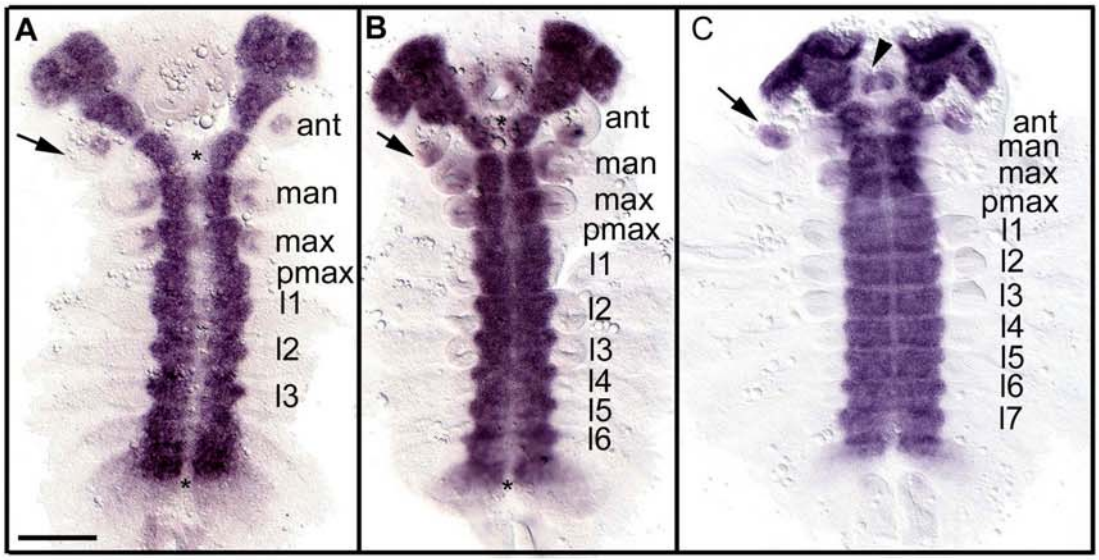


Fig. 15



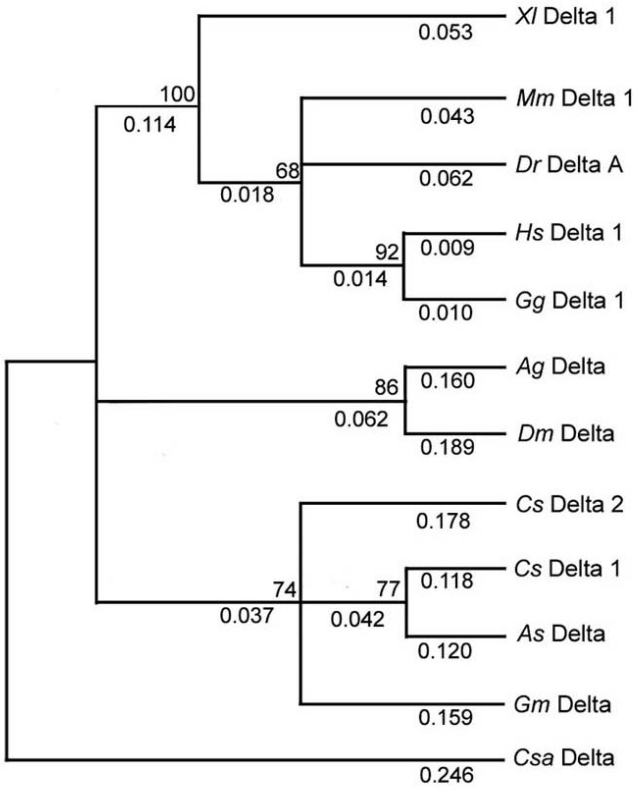
3.3.2. Neurogenic genes

While the proneural genes and the *Sox* group B genes promote neuroblast formation, the neurogenic genes *Notch* and *Delta* restrict the number of cells in the *D. melanogaster* neuroectoderm that actually enter the neural pathway. As was already mentioned in the introduction, *D. melanogaster* proneural genes are expressed in groups of four to five cells, and one cell from each cluster is then singled out to become the neuroblast by the action of neurogenic genes (Skeath and Carroll, 1992). In a similar manner, it was found in the spider *Cupiennius salei* that loss of function of neurogenic genes leads to an upregulation of the proneural gene *CsASH1* and an altered morphology of the VNE (Stollewerk, 2002). In order to compare the expression of neurogenic genes between chelicerates and myriapods, I was interested in isolating *Notch* and *Delta* homologs from *G. marginata*.

3.3.2.1. Isolation of neurogenic genes

Only one original fragment was found for the *GmDelta* and *GmNotch* genes, and larger fragments were amplified by RACE PCR. This resulted in a 2056 bp *GmDelta* sequence with an open reading frame of 685 amino acids covering a part of the N-terminal signal sequence, the DSL domain and eight EGF repeats, and a 1110 bp five prime *GmNotch* sequence with a 377 deduced amino acid sequence comprising the N-terminus and the first 12 EGF repeats. For both sequences, it was not possible to unambiguously identify the start codon.

For *Delta*, an *Archispirostreptus* sequence covering the DSL domain and EGF repeats 1 and 2 with 81% similarity to the spider CsDelta1 deduced protein sequence was isolated. The complete GmDelta putative amino acid sequence can be completely aligned with CsDelta1 and shares 58% identical amino acids. An alignment of the DSL domains of several species shows that these sequences are highly conserved. The DSL domain and EGF repeats 1 and 2 from two insect species, five vertebrates, the two myriapods, the spider *Cupiennius salei* and the ascidian sequence from *Ciona savigny* were aligned to create Tree 4. Here, the insects and the vertebrates form two clear groups, while the myriapods group with the spider sequences. All three of these groups have relatively high bootstrap support (insects: 86%; vertebrates: 100%; spider and myriapods: 74%).



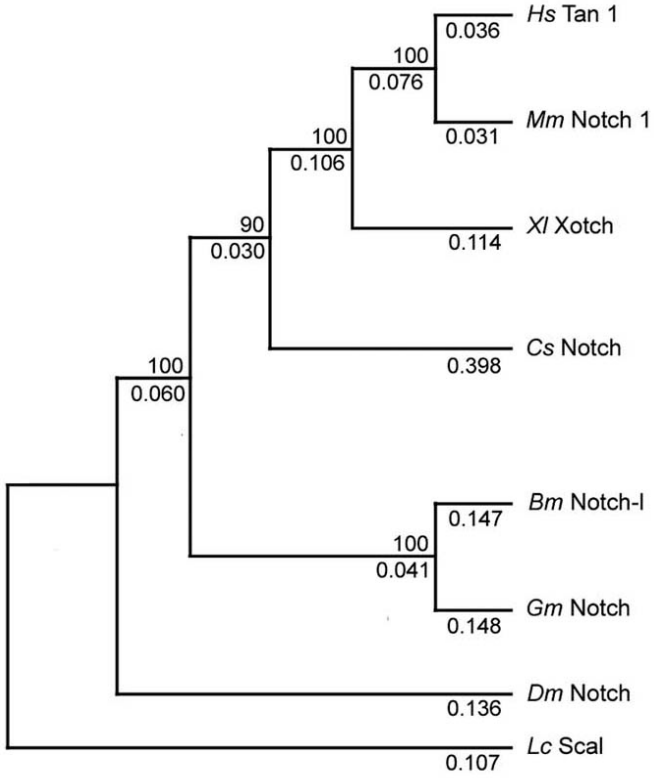
Tree 4. Phylogeny of the conserved domain of Delta. The tree was created from an alignment of the DSL domains and the adjacent highly conserved EGF-repeats 1 and 2 from two insect species, five vertebrate, two myriapod, an ascidian and the two spider sequences using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. The insects and the vertebrates form two clear groups, while the myriapods group with the spider sequences. Species: *Ag*, *Anopheles gambiae*; *As*, *Archispirostreptus spec.*; *Cs*, *Cupiennius salei*; *Csa*, *Ciona savingy; Dm*, *D. melanogaster*; *Dr*, *Danio rerio*; *Gg*, *Gallus gallus*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus; Xl*, *Xenopus laevis*.

Alignement 2 Comparision of the highly conserved DSL domain (Delta, Serrate, Lag2) of GmDelta with the DSL domains of another diplopod, two insect, two vertebrate and two spider sequences. The GmDelta DSL domain shows the highest similarity to the same protein region of *Anopheles gambiae* (67% identical amino acids), *Archispirostreptus spec*. and *Cupiennius salei* Delta1 (65% identical amino acids each). Species: *Ag*, *Anopheles gambiae*; *As*, *Archispirostreptus spec*.; *Cs*, *Cupiennius salei*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus.*

```
Dm Delta     YDFRVTCDLNYYGSGCAKFCRPRDDSFGHSTCSETGEIICLTGWQGDYCHIPKCAKG
Ag Delta     .AY..H.SV....NI.GDL......K...FN..P..AKV..P..T.E..SKAV..P.
Gm Delta     FA...R..EHH..P...VT......K...F....Q.GKV..P..T....DVAV..P.
As Delta     .A......P....A...NL..K...Q...Y...PI..RV..S..L.E..TVAQ.TP.
Cs Delta1    .AI..R.LD....ES.E.L....N.K...Y...P..DKV..R..T.E..T.AV.LP.
Cs Delta2    LMY..Y.AT....PN.GNL......K...Y..K.D.QKL.KP..S.S..DKAL.LP.
Hs Delta1    .SY.FV..EH...E..SV.......A...F..G.R..KV.NP..K.P..TE.I.LP.
Mm Delta-l1  .SY.FV..EH...E..SV.......A...F..GDR..KM.DP..K.Q..TD.I.LP.
```

The obtained *G. marginata* Notch sequence, which shares 68% of its amino acids with the *Boophilus microplus* (chelicerate) Notch homolog, was aligned with seven Notch homologs to create Tree 5. The high sequence similarity between the *G. marginata* and the *B. microplus* proteins is reflected by the tree, where the node joining the chelicerates with the vertebrates and *G. marginata* has a bootstrap support of 100.

The insect sequences, on the other hand, are joined by a node with less than 50% support.



Tree 5. Phylogeny of the conserved domain of Notch. The tree was constructed from an alignment of the obtained GmNotch sequence (5´prime region up to EGF-repeat 12) with the same region of four vertebrate and three invertebrate Notch homologs using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50 %. Numbers below the branches are the branch lengths. The myriapod sequence groups with the chelicerate *Boophilus microplus*, while the spider homolog forms a group with the vertebrates. The node joining the chelicerates with the vertebrates and the myriapod has a high bootstrap support (100 %). Genes: Scal, Scalloped wings. Species: *Bm*, *Boophilus microplus*; *Cs*, *Cupiennius salei*; *Dm*, *D. melanogaster*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Lc*, *Lucilia cuprina*; *Mm*, *Mus musculus.* *Xl*, *Xenopus laevis*.

3.3.2.2. Expression patterns of *GmDelta* and *GmNotch*

GmDelta is first expressed during the first wave of neural precursor formation at stage 2 (Fig. 16, p. 71). Transcripts can be detected at low levels in all VNE cells, but accumulate at higher levels in the invaginating cell groups, similar to the expression pattern of the spider *CsDelta2* gene. *GmDelta* is also expressed in all invagination sites generated subsequently (Fig. 16 D-F). The expression seems to be rapidly down regulated, since transcripts cannot be detected in all invagination sites generated at different waves (Fig. 16 A, B, D, F). In addition, *GmDelta* is expressed in precursors of the PNS at the base of the leg appendages and in stage 5 in groups of cells in the dorsal plates (arrow Fig. 16 B, E).

GmNotch is expressed in segmentally repeated stripes at stage 1, but shows a stronger expression in the VNE (Fig. 17, p. 71). During formation of the first invagination sites the expression in stripes becomes restricted to the dorsal part of the embryo, lateral to the limb buds (Fig. 17 D). *GmNotch* is expressed at weak levels in

almost all cells of the VNE up to leg segment 3 (Fig. 17 D). During the next wave of neural precursor formation at stage 3, there is a clear heterogeneity in the expression levels of *GmNotch* (Fig. 17 A). This expression pattern is maintained during subsequent waves of neural precursor formation. *GmNotch* expression extends to more posterior segments during the course of neurogenesis. As in the anterior segments, the expression is uniform during the first wave of neural precursor formation and shows heterogenous expression during formation of the remaining invagination sites.

In summary, the data show that the *G. marginata* homologs of the *D. melanogaster Notch* and *Delta* genes are expressed in the VNE during neurogenesis in a spatio-temporal pattern comparable to the spider, suggesting that these genes are involved in the recruitment of neural precursors (Stollewerk, 2002).

Figure Legends 16 and 17

Fig. 16 (A - F) Expression pattern of *GmDelta*. Flat preparations of whole embryos (A, B) and leg segments (C - F) stained for a DIG-labeled *GmDelta* probe. (A, B) GmDelta transcripts can be detected at weak levels in all ventral neuroectodermal cells, but accumulate at higher levels in the invaginating cell groups. In addition, the gene is expressed in groups of cells in the limb buds and dorsal to the limb buds (arrow heads). These regions coincide with the generation sites of the peripheral nervous system. (C) Accumulation of higher levels of *GmASH* transcripts is first visible during formation of the first invagination sites at stage 2 (arrow). (D - F) High levels of *GmASH* expression correlate with the formation of invagination sites throughout neurogenesis (arrows). The expression is rapidly downregulated during the process of invagination, although the low uniform expression in all neuroectodermal cells is maintained. *ant*, antennal segment; *l1* to *l6*, leg segments 1 to 6; *man*, mandibular segment; *max*, maxillar segment; *pman*, premandibular segment; *pmax*, postmaxillar segment. *l1* to *l6*, leg segments 1 to 6. Scale bars: 120 µm in A, B; 25 µm in C - F.

Fig. 17 (A - F) Expression pattern of *GmNotch*. Flat preparations of whole embryos (A, B) and leg segments (C - F) stained for a DIG-labeled *GmNotch* probe. (A, B) At stage 4 *GmNotch* is expressed in all neuroectodermal cells (arrows) at heterogenous levels. (C) At stage 1 *GmNotch* is expressed in segmentally repeated stripes, but shows a stronger expression in the ventral neuroectoderm (arrow). (D) During the first wave of neural precursor formation, *GmNotch* is expressed uniformly in the head segments and the first three leg segments (arrow). (E) The uniform expression resolves into a heterogenous expression pattern before formation of the next wave of invagination sites. The arrow head points to a region of low *GmNotch* expression, a higher expression is visible in the adjacent region (arrow). (F) *GmNotch* expression has extended posteriorly and still shows a heterogenous expression pattern in the neuroectoderm. The expression in the limb buds probably corresponds to the formation of mesodermal tissue (arrow head). *ant*, antennal segment; *gz*, growth zone; *l1* to *l5*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pman*, premandibular segment; *pmax*, postmaxillar segment. Scale bars: 120 µm in A, B; 25 µm in C - F.

Figures 16 and 17

Fig. 16

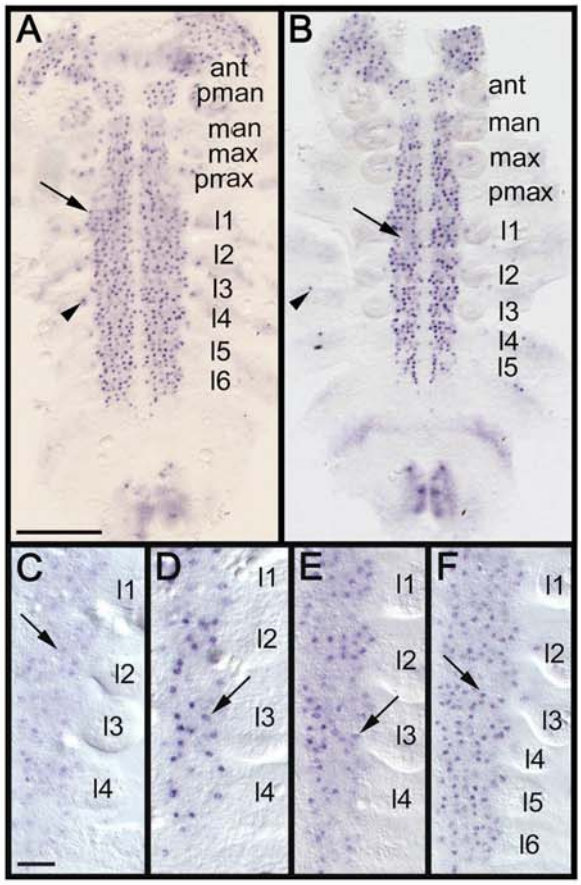
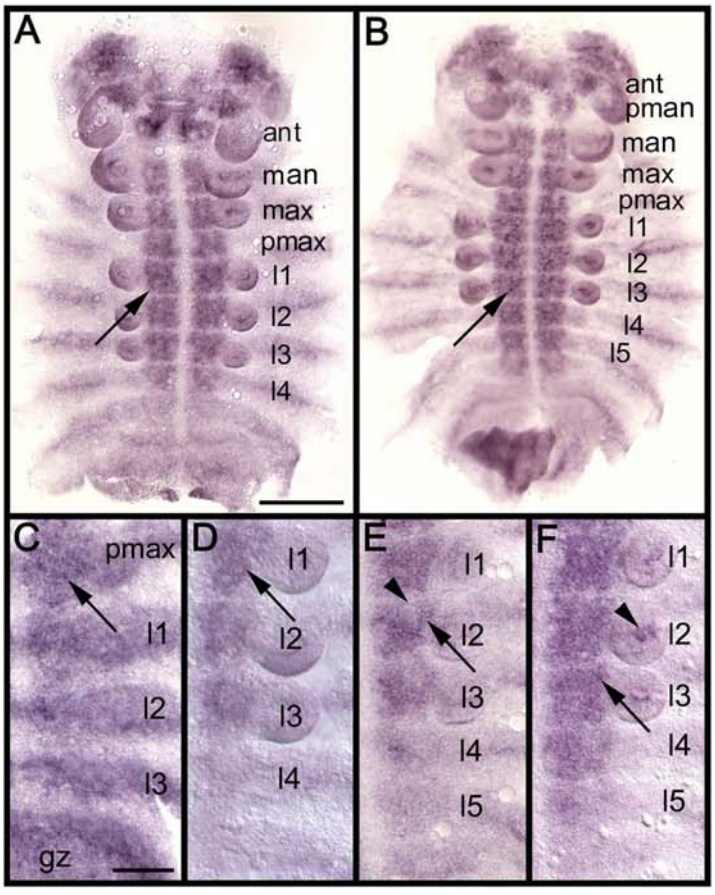


Fig. 17



3.3.3. Dorso-ventral patterning of the ventral neuroectoderm

As was mentioned in the introduction, the *D. melanogaster* VNE is patterned along the dorso-ventral axis by three homeobox genes: *muscle segment homolog* (*msh*), *intermediate neuroblasts defective* (*ind*) and *ventral neuroblasts defective* (*vnd*). These genes are expressed in the lateral, intermediate and medial regions of each hemisegment, respectively, as an antero-posterior stripe and together with the AP-patterning genes, these constitute a Cartesian cell-fate determination system for the developing CNS (for a review see Skeath, 1999). I investigated whether this subdivision into three columns of gene expression of the dorso-ventral patterning genes can be found in myriapods.

3.3.3.1. Isolation of patterning genes

Only one original fragment was identified for *GmInd* and *GmMsh*. For *GmMsh*, 5' RACE resulted in a 1040 bp fragment with an ORF of 9 - 1040. While an upstream stop codon was found, no unambiguous start codon could be identified. The putative amino acid sequence shows the highest similarity to the Msx protein from *Brachistoma floridae* (68%) and the vertebrate Msh homologs and 60% identity to the *D. melanogaster* protein.

For *GmInd*, RACE primers were designed to amplify the 3' end of the protein. Since the fragments obtained were relatively short and the RNA expression patterns observed by in-situ hybridisation were weak, new 5' primers were designed. The expression patterns observed with an in-situ probe from the larger fragment isolated, however, were equally weak. The complete fragment is 770 base pairs long and has an ORF from 2 - 573. An unambiguous downstream stop codon could be identified, but, while the length of the protein is consistent with data from similar proteins in other species, no upstream stop codon was identified. The fragment shows 90% similarity to the *Mouse GSH-1* in the homeobox domain (the rest does not align at all) and is 82% identical to the *D. melanogaster intermediate neuroblasts defective* gene.

In the case of *vnd*, primers were designed based on the highly conserved *Nk2* type homeobox, but unfortunately the fragment obtained by RACE PCR was a *tinman* homolog rather than *vnd*. Thus, it was not possible to study *vnd* as a part of this

research, but it would certainly be worthwhile to start a second attempt at obtaining a true *vnd* homolog by taking the *tinman* sequence into account (sequence in appendix).

3.3.3.2. Expression patterns of *GmMsh* and *GmInd*

Both *GmMsh* and *GmInd* expression begin as soon as the segmented embryo is visible around stage 1 and persist throughout embryonic development (Fig. 18, 19 and 20, p. 77). At stage 1, *GmMsh* RNA is visible in the most lateral part of the VNE in all segments, and additionally as segmentally repeated dorso-ventral stripes in leg segments 2 to 5 and in the growth zone (Fig. 18, A). The expression in the antennal segment forms a triangle at the base of the appendage, which can be compared to the expression in the lateral VNE of the other segments (Fig. 18 A arrow). This lateral expression is very strong and is present in all segments before the appendages and invagination sites are formed. Weaker expression is visible in the positions where appendages will form, and in a broad domain in the dorsal plates.

As each additional leg segment is formed in the course of development, *msh* expression begins and persists in the regions described above (Fig. 18 B, C, D, E). Beginning at stage 4, expression is also visible in a group of cells in the most medial part of each hemisegment (Fig. 18 D, E, arrows in H, I). It is interesting to note that this is the area where the last invaginations form. Further analysis of expression in the right hemisegment of the first leg segment shows that, intitially, *msh* is visible in a group of cells in the lateral part of the hemisegment and in an area that will probably form the leg appendage (Fig. 20 A, arrow).

In stage 2, a stripe of expression is present in the most lateral part of the hemisegment and a group of cells expressing *msh* mark the position where the leg will form (Fig. 20 B arrow). From stage 3 onwards, expression is strongest in the most posterior part of the lateral stripe (Fig. 20 C; 18 G arrow), and the additional medial expression is visible from stage 4 onwards (Fig. 20 D arrow; 18 H, I arrows). Thus, it can be said that throughout development *GmMsh* is expressed in the most lateral part of each hemisegment before, during and after invagination site formation.

Compared to the strong *GmMsh* expression, *GmInd* expression is very weak and restricted to a small group of cells in the middle of each hemisegment. *GmInd* expression is, however, specific and becomes visible as each leg segment forms (Fig. 19 A, B, C, arrows). Detailed comparison of *GmInd* expression in the first leg segment

(Fig. 20 E - H) to *GmMsh* expression (Fig. 20 A - D) showed that it is expressed in a more medial domain that most likely does not overlap with *GmMsh*.

Thus, in *G. marginata*, as in *D. melanogaster*, *msh* and *ind* are expressed in the VNE, in lateral and intermediate domains, respectively, but there are some significant differences to expression in other species, such as the additional medial expression of *GmMsh.*

Figure Legends 18 - 20

Fig. 18 (A - I) Expression pattern of *G. marginata msh*. Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A) At stage 1, *GmMsh* RNA is visible in the most lateral part of the VNE in all segments, and additionally as a stripe connecting the left and right hemisegments in leg segments 2 to 5 and in the growth zone. The arrow points to the expression in the antennal segment, which forms a triangle at the base of the appendage. This can be compared to the expression in the lateral VNE of the other segments. Lateral expression is very strong and is present in all segments before the appendages and invagination sites are formed. Weaker expression is visible in the positions where appendages will form, and in a broad domain in the dorsal plates. (B - E) As each additional leg segment is formed in the course of development, *msh* expression begins and persists in the regions described above (B, F) Stage 2 embryos. The arrow in F points to the expression where the appendage will form. (C, G) Stage 3 embryos. The expression in the dorsal plates is shown by the arrow in C. The arrow in G points to the strongest *msh* expression in the hemisegment. (D, H) At stage 4, additional expression becomes visible in the middle of each hemisegment, as shown in H by the arrow. (E, I) Stage 5 embryos continue to express *msh* in the regions described. The arrow in I points to the additional expression in the middle of the hemisegment. *ant*, antennal segment; *gz*, growth zone; *l1* to *l7*, leg segments 1 to 7; *man*, mandibular segment; *max*, maxillar segment; *pmax*, postmaxillar segment. Scale bars: 120 µm in A - E; 50 µm in F - I.

Fig. 19 (A - F) Expression pattern of *G. marginata ind*. Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A, D arrow) Stage 2 embryos. Expression is visible in a group of cells in the middle of each hemisegment in the postmaxillar segment and leg segments 1 to 3. No expression is visible in the midline. (B, E) In stage 4 embryos three additional leg segments have been formed by the posterior growth zone and express *GmMsh* in a group of cells in the middle of each hemisegment (C) This expression is still present in late stage 5 embryos. *ant*, antennal segment; *l1* to *l5*, leg segments 1 to 5; *man*, mandibular segment; *max*, maxillar segment; *pmax*, postmaxillar segment. Scale bars: 120 µm in A - C; 50 µm in D - F.

Fig. 20 (A - H) Comparison of the expression patterns of *G. marginata msh* (A - D) and *ind* (E - H) in one hemisegment of the first leg segment. The midline is to the left and anterior is towards the top. Asterisks denote the probable relative position of the expression of the other gene in the hemisegment. (A) At stage 1, *GmMsh* RNA is visible in the most lateral part of the hemisegment. The arrow points to expression in an area that will probably form the leg appendage. (B) In stage 2, a stripe of expression is present in the most lateral part of the hemisegment and the arrow points to a group of cells expressing *msh* that mark the position where the leg will form. The asterisk denotes the probable relative position of *GmInd* expression. (C) After stage 3, *GmMsh* expression is strongest in the most posterior part of the lateral stripe. (D) From stage 4 onwards, additional medial *GmMsh* expression is visible as shown by the arrow. (E - H) *GmInd* expression in stages 1 - 4, respectively, is restricted to a small cluster of cells in the middle of the hemisegment. Scale bar: 10 μm in A - H.

Figures 18 - 20

Fig. 18

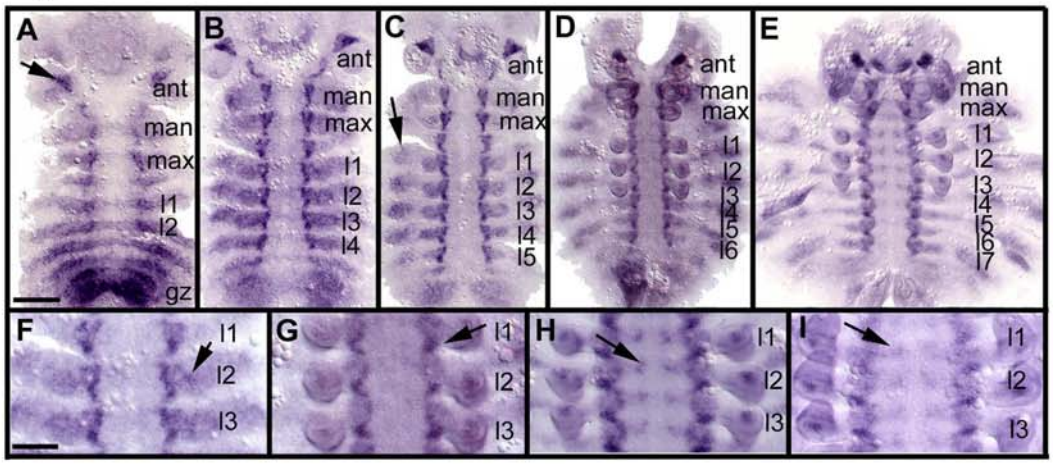


Fig. 19

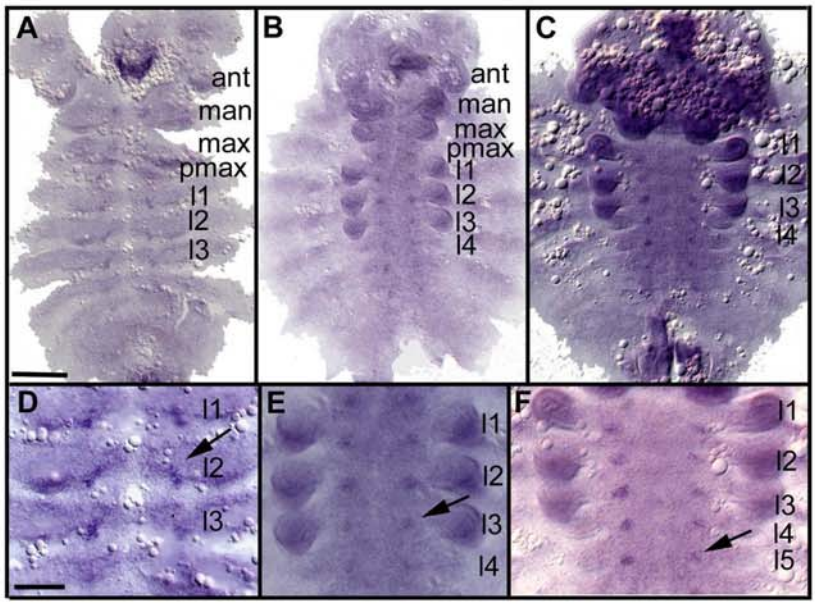
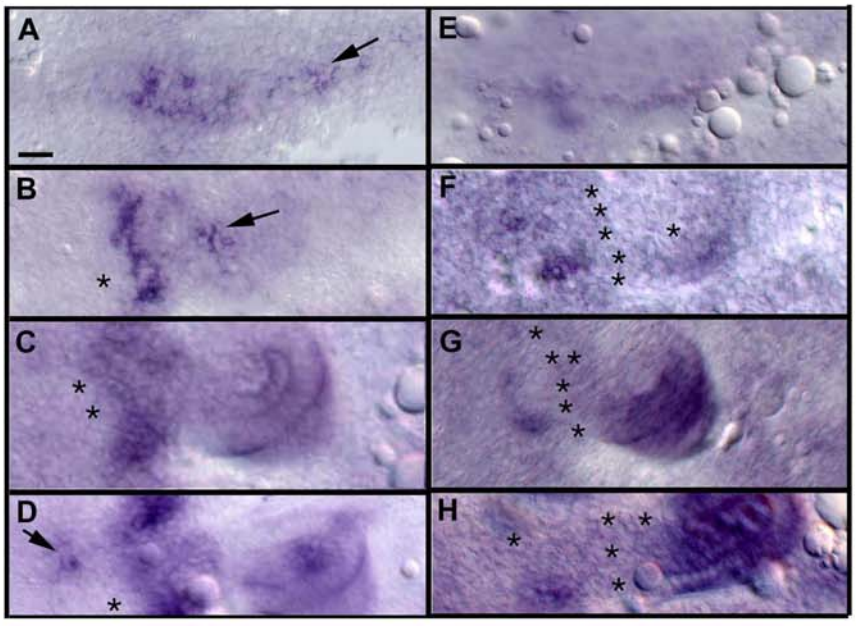


Fig. 20



3.3.4. Markers of cell fate specification

In *D. melanogaster* several genes are responsible for the generation of the ganglion mother cells via asymmetric cell division. As discussed in the introduction, this includes *numb*, *prospero* and the *snail* genes in *D. melanogaster* (reviewed in Broadus and Spana, 1999). Since stem cells may be present in the VNE of *G. marginata*, in contrast to the spider, I was interested in the distribution of *snail* and *prospero.* In addition, *islet* is a marker for a subset of motor- and interneurons in *D. melanogaster*. I was interested in recording the expression of these genes for later comparison to the data obtained from *Cupiennius salei.*

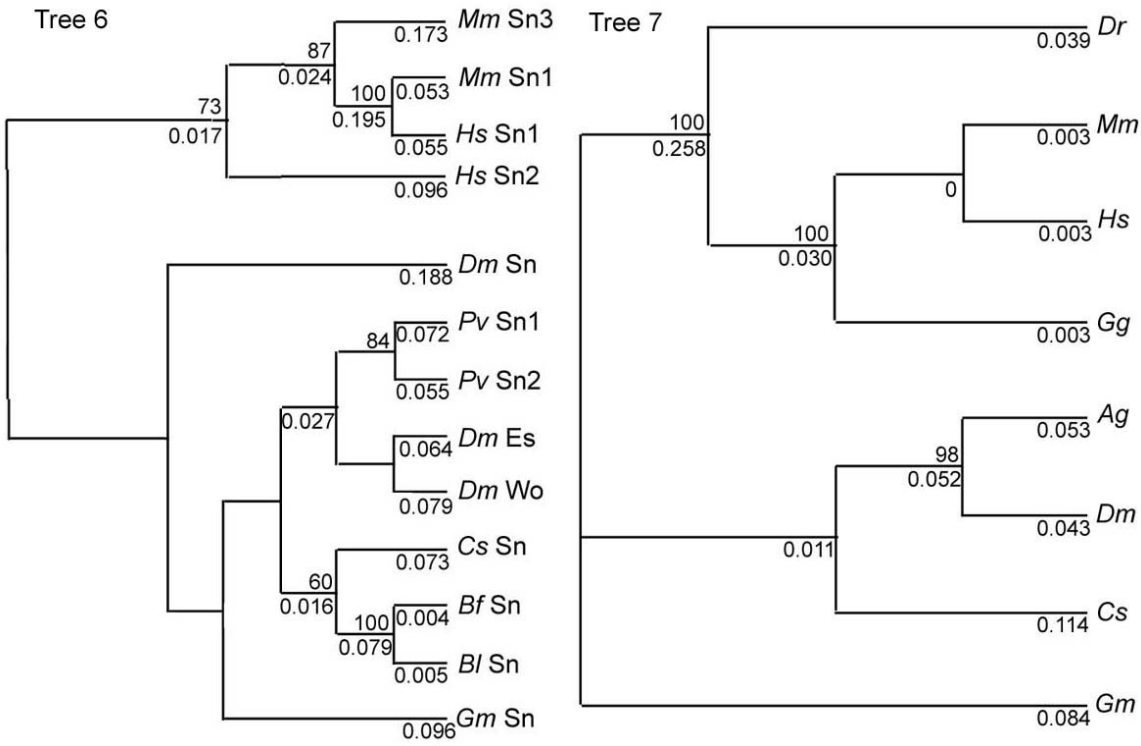
3.3.4.1. Isolation of *G. marginata snail*

Only one *snail* homolog was identified for *G. marginata*, and 3' RACE yielded a 862 base pair fragment with an open reading frame of +1 and 81% blast homology to both *D. melanogaster* and *Cupiennius* Snail. The fragment does not have an upstream stop codon and ends in the middle of the fourth zinc finger. One conserved P-DLS-K motif could be identified (amino acids 21 - 27) that is also found in other *snail* homologs and is known to interact with the dCtBP co-repressor in *D. melanogaster* (Ashraf *et al.*, 1999). It is probable that neither the 3' nor the 5' end have been identified. Nevertheless, an alignment starting at the first zinc finger excluding gaps led to a tree that shows that the vertebrate sequences group together with relatively high bootstrap support (73%), but that the bootstrap support for the grouping of the other species is relatively low (Tree 6). *G. marginata snail* does not group with any other sequences with high bootstrap support.

3.3.4.2. Isolation of *G. marginata prospero*

All primary *prospero* clones were identical, and 5' RACE yielded a 720 base pair fragment with an open reading frame of 2 - 615 and a 3'UTR. The putative amino acid sequence is 73% identical to the *D. melanogaster* and 67% to the *Cupiennius salei* Prospero protein. The *GmPros* RACE sequence encompasses the homeodomain and the *prospero* domain, but since no 5` RACE was performed, nothing can be said about the

nuclear localisation signal or the asymmetry domain. An alignment of the amino acids 41 - 200 with the corresponding regions of other prospero homologs indicates that the *G. marginata* sequence groups with the invertebrates rather than the vertebrates; however, this grouping does not show high bootstrap support in Tree 7. The vertebrate sequences group together with 100% support, as do the two insect sequences, but *G. marginata* and *C. salei* Prospero do not group together or with any of the other sequences.



Trees 6 & 7 Phylogeny of the conserved domains of Snail and Prospero homologs. The trees were constructed using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. **Tree 6**. The vertebreate sequences group together with high bootstrap support, but none of the other larger groupings are significant. *G. marginata* and *Cupiennius* snail are both relatively derived as can be seen from their branch lengths. **Tree 7.** The vertebrate and insect sequences form two distinct groups with high bootstrap support, but the spider and millipede sequences do not group together or with any other Prospero homologs. Genes: Sn, Snail; Es, Escargot; Wo, Worniu; vertebrate Prospero sequences are Prox1. Species: *Ag, Anopheles gambiae; Bf, Brachistoma floridae; Bl*, *Brachistoma lanceolata*; *Cs*, *Cupiennius salei*; *Dm*, *D. melanogaster*; *Dr, Danio rerio*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus; Pv, Patella vulgata.*

3.3.4.3. Expression of *snail* and *prospero*

G. marginata snail is first expressed in stage 1 embryos in groups of cells in the head segments and in two groups of cells in the most medial part of each hemisegment of the postmaxillar and leg segments 1 - 3 (Fig. 21 A, p. 82). By early stage 2, this expression is visible in leg segment 4 and the head and leg segments 1 - 3 show a column of expression on either side of the midline, as well as dots of expression in the invagination sites (Fig. 21 B; 22 A, p. 82). Expression in cell clusters in the head persists throughout embryonic development. At early stage 3, *snail* is expressed as a stripe in the midline except at the segment borders, and where invagination sites have formed (Fig. 21 C; 22 B). By late stage 3, the complete midline expresses *snail* and expression persists in the invagination sites (Fig. 21 D; 22 C). In stages 4 and 5, spots of expression are added as invagination sites are formed in leg segments 4 - 7 (Fig. 21 E, F; 22 D, E). *Snail* appears to be expressed in every invagination site in the VNE after it has formed as well as in the head and the midline.

Prospero, on the other hand, is not expressed in the ventral midline. Like *snail*, expression is visible in all invagination sites, but expression levels are higher in the most medial invagination sites and there even appears to be weak staining in the most medial cells that are not part of the invagination sites. At stage 3, expression is visible in all invagination sites present in leg segments 1 - 4 and in the head segments, comparable to the *snail* expression in the VNE (Fig. 21 H). By stage 4, expression is visible in some invagination sites in leg segment 4, and, reminiscent of early *snail* expression, in two medial clusters of cells in each hemisegment of leg segment 5 (Fig. 21 I; 22 F, H). Additionally, groups of cells at the tips of the head appendages express *prospero*. Expression in the invagination sites and in the head appendages persists through stage 5, by which time all invagination sites express *prospero* and *snail*, though *prospero* expression continues to be stronger in the most medial invagination sites (Fig. 21 I; 22 G, I). At this stage additional expression is present in clusters of cells in the dorsal plates, which may correspond to the positions of nascent sensory organs of the PNS (Fig. 22 I arrows).

To summarize, *snail* and *prospero* are both expressed in groups of neural precursor cells after they have formed. *Snail* is also expressed in the midline, where *prospero* is absent, while *prospero* shows specific expression in the PNS and stronger expression in the most medial invagination sites.

Figure Legends 21 and 22

Fig. 21 (A - I) Expression patterns of *G. marginata snail* (A - F) and *prospero* (G - I). Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A) In stage 1 embryos, *snail* is expressed in groups of cells in the head and in two medial groups of cells, as shown by the upper arrow. (B, C) By the end of stage two, invagination sites in the head and leg segments 1 to 4 express *snail*, and there is expression in the ventral midline. (D) In stage 3 embryos *snail* is expressed in the complete midline and invagination sites in the head and leg segments. (E, F) As invagination sites are formed in stages 4 and 5, they begin to express *snail*. Expression persists in the head and leg segments as well as in the midline. (G, H) Stage 3 and 4 embryos, respectively, express *prospero* in all invagination sites, comparable to *snail* expression, but stronger expression is visible in the most medial invagination sites. (I) As invagination sites are formed on leg segments 4 to 6, they begin to express prospero. The expression in the head and in the VNE remains. *l1* to *l4*, leg segments 1 to 4; *man*, mandibular segment; *max*, maxillar segment; *pmax,* postmaxillar segment. Scale bar: 120 µm in A - I.

Fig. 22 (A - I) Closeup of *G. marginata* embryos stained for *snail* (A - E) or *prospero* (F - I) RNA. Anterior is to the top. (A) Dots of *snail* expression in regions where the first invagination sites have formed in leg segments 1 and 2 in early stage 2 embryos. *Snail* is also expressed in the midline. (B) In leg segments 1 and 2, further dots of high *snail* expression are visible. The first *snail* expressing cell groups have formed in leg segment 3. (C) In late stage 3 embryos, all invagination sites of leg segments 1 - 3 express *snail*. Invagination sites are visible in leg segments 4 and 5. (D) By late stage 4, *snail* is expressed in all invagination sites of leg segments 1 - 5. (E) Leg segments 1 - 7 express *snail* in groups of cells in the VNE. (F, G) *Prospero* expression is visible in groups of cells in the head appendages of stage 4 and 5 embryos, respectively. (H) Expression of *prospero* in invagination sites of stage 3 embryos can be compared to *snail* expression in (C). While both genes are expressed in all invagination sites, *prospero* expression is stronger in the more medial invagination sites. (I) Additionally, *prospero* is expressed in clusters of cells in the dorsal plates which may correspond to precursors of the PNS as shown by the arrows. *l1* to *l7*, leg segments 1 to 7; *man,* mandibular segment; *max*, maxillar segment. Scale bar: 50 µm in A - I.

Figures 21 and 22

Fig. 21

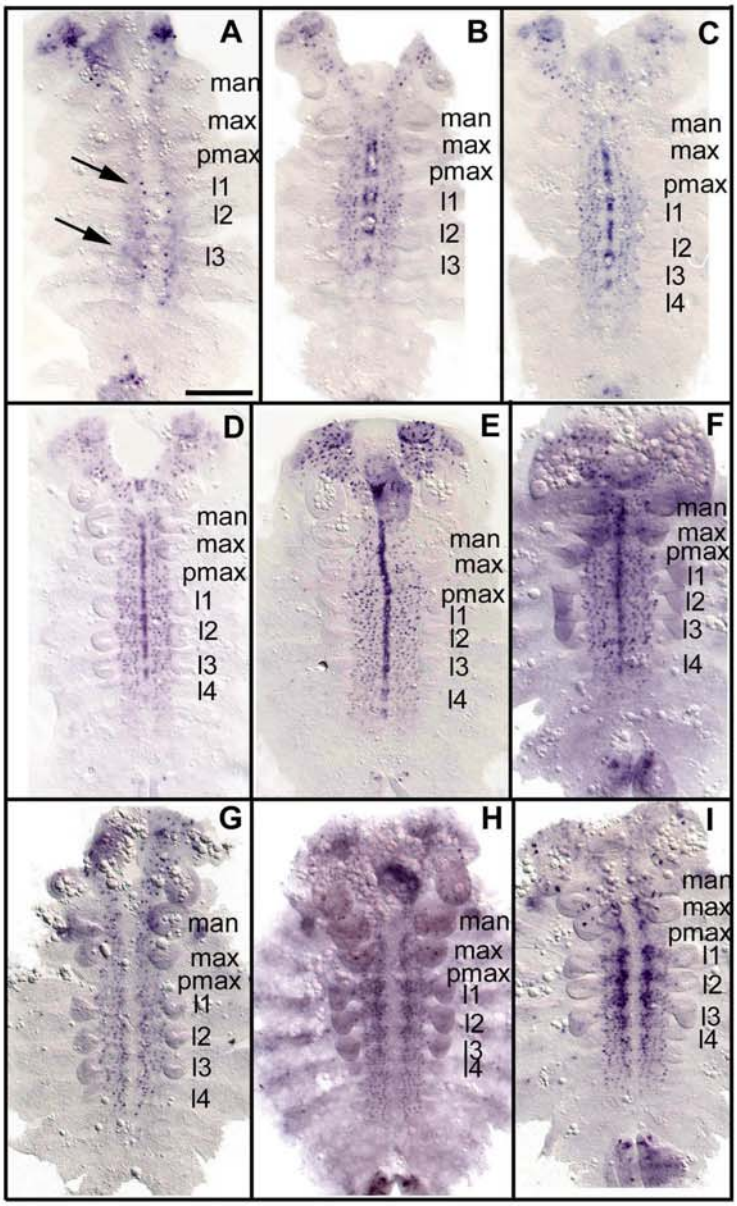
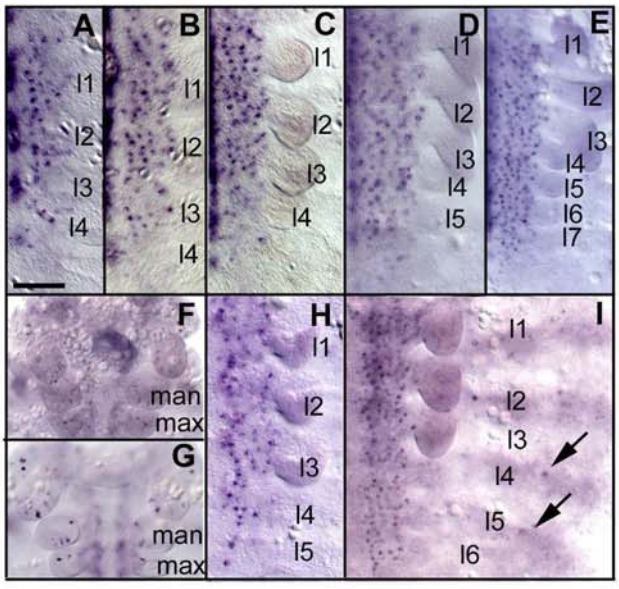
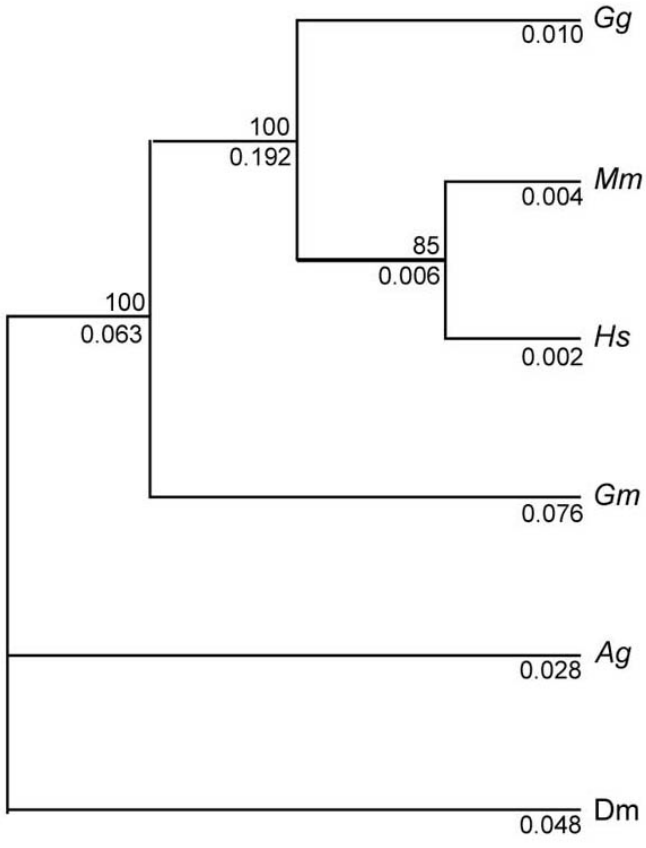


Fig. 22



3.3.4.4. *G. marginata numb* homolog

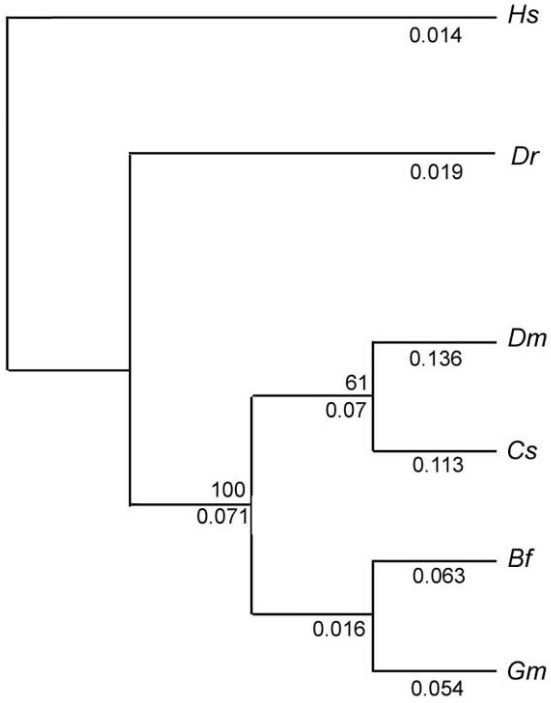
Three prime RACE led to the isolation of two fragments (transcripts 1 and 2, see alignment in appendix), which are identical except that there is a region 5' of the zinc finger domain missing in one of the two fragments. The *G. marginata* transcripts are 938 and 929 bp and the shorter of the two transcripts is missing 80 amino acids (240 nucleotides) but has a longer coding sequence. No stop codon was found, so it is not clear whether the 3' end was identified. Both transcripts have the highest similarity to the *Anopheles gambiae* sequence XP_319339.1 (74%) and 68% similarity to the *D. melanogaster* Numb protein overall; however, an alignment of the conserved numb zinc finger (excluding gaps) led to Tree 8, which shows that the *G. marginata numb* fragment groups with the vertebrate sequences with 100% bootstrap support, while the *A. gambiae* and *D. melanogaster* sequences do not group together and may thus be highly derived. It would be interesting to isolate the 5' RACE sequence to see if different transcripts can be identified. In-situ hybridisation with *numb* RNA probes show that both transcripts are expressed ubiquitously in the *G. marginata* embryo, but not in yolk cells, from stage 1 to stage 5 of development.



Tree 8. Phylogeny of the conserved zinc finger domain of Numb homologs of two insect, three vertebrate, and the myriapod sequences using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. The myriapod numb sequence groups with the vertebrates, while the insect sequences do not group together. Species: *Ag*, *Anopheles gambiae*; *Dm*, *D. melanogaster*; *Gg*, *Gallus gallus*; *Gm*, *G. marginata*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus.*

3.3.4.5. *G. marginata islet* homolog

An 869 bp *islet* fragment was isolated, with an ORF of 1 - 869. The putative amino acid contains the LIM homeobox domain and is 54% similar to the *D. melanogaster* Tailup/Islet protein, 72% to the vertebrate Islet proteins and 70% identical to the *C. salei* homolog (Stollewerk, unpublished data). As is the case for *D. melanogaster*, only one *G. marginata islet* homolog was identified with equally high similarity to both vertebrate homologs. An alignment of the conserved LIM homeoboxes led to Tree 9, which shows that the invertebrate sequences group together with 100% bootstrap support, while the relationships within the invertebrates are not highly supported.



Tree 9. Phylogeny of the conserved Islet LIM homeoboxes of one insect, two vertebrate, one chelicerate, one cephalochordate and the myriapod using the neighbour-joining algorithm (see Materials and Methods). Numbers at the nodes are the bootstrap values given in percent (1000 replicates). Nodes without numbers have bootstrap values below 50%. Numbers below the branches are the branch lengths. The invertebrate sequences group together, however the relationships within this group are unclear. Only one mammalian islet homolog is shown here because the mammalian islet genes are almost identical. In the case of the vertebrates, several islet homologs are known, but only Islet 1 was used for this tree. Species: *Bf, Brachistoma floridae*; *Cs, Cupiennius salei*; *Dm*, *D. melanogaster*; *Dr, Danio rerio; Gm*, *G. marginata*; *Hs*, *Homo sapiens*.

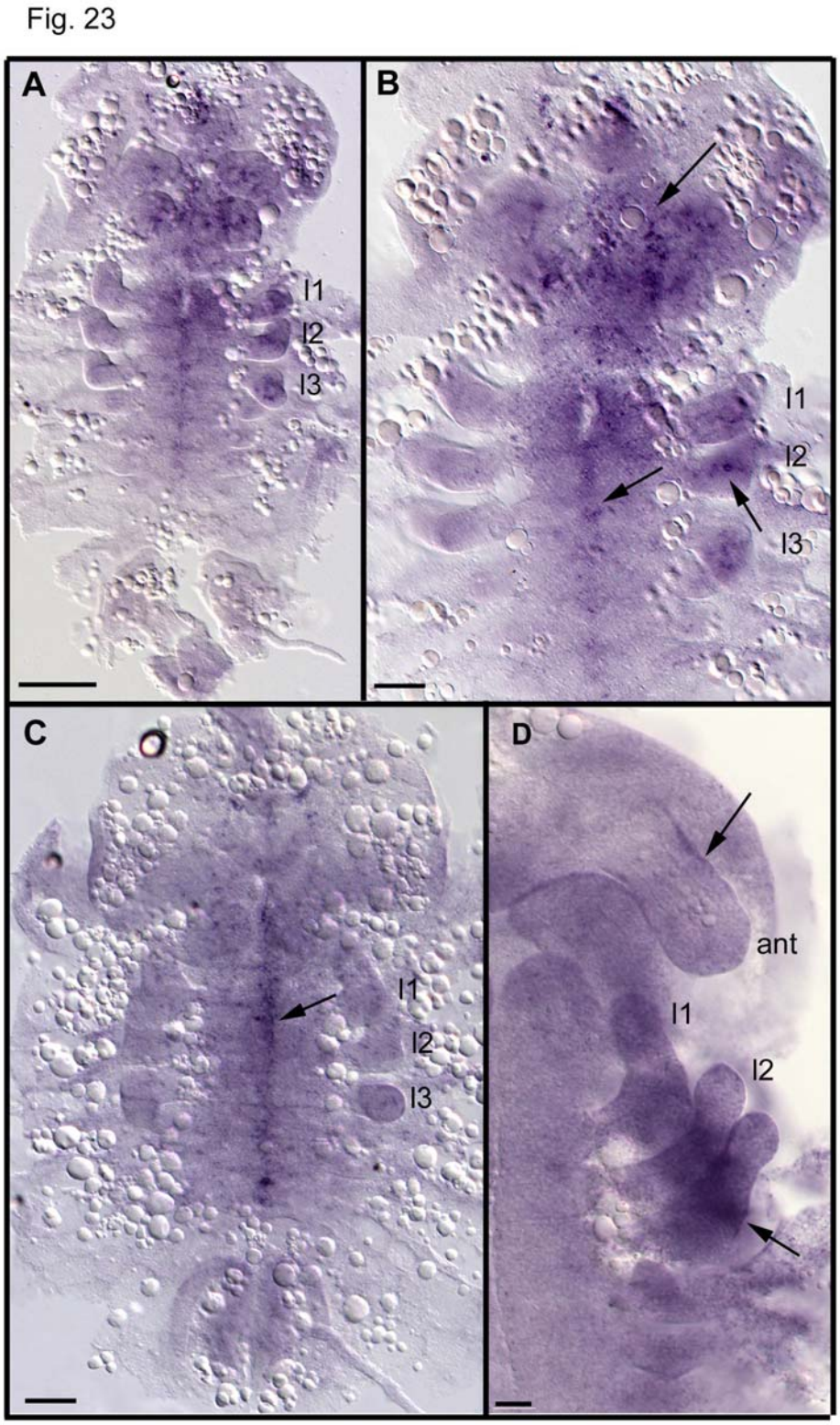
3.3.4.6. Expression of *G. marginata islet*

Islet expression is first seen very late in development in stage 5 embryos, where it is expressed in the ventral midline and in groups of cells in the head (Fig. 23 A, B arrows, p. 86). In stage 6 embryos, an additional stripe of expression can be seen in the leg and head appendages (Fig. 23 C, D arrows), however, since it is very difficult to make flat preparations of embryos in this stage of development, i*slet* expression could not be studied on the whole mount. Further studies of sections will show if *islet* is expressed in specific neurons.

Figure Legend 23

Fig. 23 (A - D) Expression of *G. marginata islet*. Flat preparations of whole embryos stained for a DIG-labeled probe. Anterior is towards the top. (A, B) Late stage 5 embryo. Arrows denote expression in groups of cells in the head, in the ventral midline and possibly in the legs. (C, D) In stage 6 embryos the expression in the head is no longer visible, but the expression in the midline is still apparent (arrow in C). Clear stripes of islet expression are visible in legs 1 - 3 and possibly in the antennae (arrows in D). *ant*, antennal segment; *l1* to *l4*, leg segments 1 to 4. Scale bars: 120 µm in A; 50 µm in B, C; 25 µm in D.

Figure 23



4. Discussion

4.1. *Glomeris marginata* as a model system

From a phylogenetic point of view, it is important to have a myriapod model system, and *G. marginata* proved to be a good study organism in which the processes of early neurogenesis can be examined. It curves inwards relatively late, compared to other diplopod species (Dohle, personal communication), allowing for detailed studies of the VNE. In addition, it is possible to obtain large numbers of eggs for RNA isolation, antibody stainings and in-situ hybridisations. Injection of live eggs after the germband has formed is possible; the embryos continue to develop normally for several days, as I showed for the BrdU staining.

So far, however, there are three major disadvantages to using *G. marginata* as a model system. First, eggs are only available three months of the year, and although this time of availability can be prolonged for several weeks, it was not possible so far to have a year-round culture. Second, the long generation time makes creation of transgenic animals impractical and, third, so far, no functional RNA-inhibition data could be obtained because it was not possible to inject embryos early enough to observe specific phenotypes. However, RNA expression patterns indicate that the genes presented here may play a similar role in neurogenesis as their homologs in other species.

This research was primarily conducted with the intention of comparing the results to data obtained from the spider, where functional RNA-i studies are possible (work that is still in progress, Stollewerk, personal communication). In some cases, therefore, the significance of the expression patterns cannot be fully discussed; however the morphological data can be directly compared to what is known from *C. salei*.

4.2. The pattern of invagination sites is strikingly similar to the spider

My results show that, similar to the spider, groups of cells invaginate from the VNE of *G. marginata*. Number and arrangement of the invagination sites are strikingly similar to the spider pattern. Both the spider and *G. marginata*, have approximately 30 invaginating cell groups arranged in a regular pattern of seven rows consisting of four to five invagination sites each. In addition, in both species the invaginating cell groups are generated in four waves (Stollewerk *et al.*, 2001; Dove and Stollewerk, 2003).

In contrast, in insects about 25 neuroblasts are generated per hemisegment that delaminate as individual cells from the VNE in five waves. The first two populations of neuroblasts are arranged in three longitudinal columns and four rows per hemisegment. This regular pattern is lost after delamination of the next population of neuroblasts because earlier born neuroblasts are shifted into a more basal position (Goodman and Doe, 1993). In summary, this data shows that the pattern of neural precursors and their mode of generation in *G. marginata* are more similar to the spider than to the insects.

However, some special features in the millipede are different from spider neurogenesis. After formation of the first invagination sites, the VNE forms a multi-layered structure of small cells, while in the spider there is only one single cell layer. This morphological difference is due to limited space in the VNE; the invagination sites are located closer together and come to lie over and above each other. In addition, the invagination sites in *G. marginata* consist of up to 11 cells as compared to a maximum of nine in the spider and do not all occupy a basal position. Only some of the invaginating cells have the typical bottle-like shape seen in the spider, so that their cell processes cover larger apical areas, and the dots of high phalloidin staining appear bigger in the millipede. In addition, while up to eleven cells contribute to an individual invagination site in *G. marginata*, only five to nine cells were counted in the spider (Stollewerk *et al.,* 2001; Dove and Stollewerk, 2003).

Furthermore, although the pattern of the invagination sites is very similar in the spider and *G. marginata*, the relative timing of generation of individual invagination sites is different. While in the spider the first invagination sites arise in the most antero-lateral region of the hemisegments, in the millipede the first invaginating cell groups are visible in the middle of the hemisegment.

In the spider, the next wave of invagination sites generates invaginating cell groups in coherent medial and posterior regions abutting the former generation sites. In contrast, in *G. marginata* newly formed invagination sites are distributed all over the hemisegement. Furthermore, the two most anterior-lateral invagination sites, which occupy strikingly similar positions in the spider and both species of millipede, are generated during the first wave of invaginations in the spider, while they are not visible until the third wave of neural precursor formation in *G. marginata* (Stollewerk *et al.*, 2001; Dove and Stollewerk, 2003).

Nevertheless, the generation of neural precursors at stereotyped positions seems to be an ancient feature that has been maintained throughout the evolution of arthropods.

4.3. Generation of neural precursors is associated with cell divisions

Studies of neurogenesis in different representatives of all myriapod groups have failed to reveal stem cell-like cells with the characteristics of insect or crustacean neuroblasts (Heymons, 1901; Tiegs, 1940; Tiegs, 1947; Dohle, 1964; Whitington *et al.*, 1991). It is assumed that neurons are produced by a generalized proliferation of the VNE. However, Knoll (1974) proposed that neuroblasts are present in the apical layer of the VNE in the centipede *Scutigera coleoptrata* generating vertical columns of neurons, a mode of neural precursor formation that would be very similar to the crustacean pattern. Analysis of neurogenesis in another centipede, *Ethmostigmus rubripes*, led Whitington and co-workers to the assumption that neural precursors with the characteristics of insect neuroblasts are absent in this species (Whitington *et al.*, 1991). They could not detect sites of concentrated mitotic activity or dividing cells that are significantly larger than the surrounding cells. Similar results have been obtained for the spider: scattered mitotic cells that do not prefigure regions where neural precursors form are distributed over the neuroectoderm (Stollewerk *et al.*, 2001).

My analysis of the mitotic pattern in the VNE of *G. marginata* with the marker anti-phospho-histone 3 revealed that dividing cells are associated with invaginating neural precursors. Furthermore, groups of dividing cells seem to prefigure the regions where invagination sites arise. In contrast to the results from *Ethmostigmus*, the dividing cells are significantly larger in size than the surrounding cells in the millipede.

To confirm these results, I developed a method for labelling mitotically active cells in live embryos with BrdU. I was interested in whether all cells of an invagination

site are descended from one dividing precursor in the VNE. Unfortunately, I did not have enough embryos to make the time series required to answer this question. While patterns of cell division in the VNE could be seen by BrdU labelling, it could not be determined whether labeled cells are associated with invagination sites. However, groups of cells at different apical-basal positions, that may represent invaginating cell groups, could be identified. Therefore, I assume that stem cell-like cells are present in the apical layer of the VNE in *G. marginata,* although this assumption has to be confirmed by single cell labelling experiments.

Since the dividing cell groups are present before formation of the invagination sites, they are different from insect and crustacean neuroblasts. In *D. melanogaster* the neuroblasts do not divide until delamination from the outer layer. In contrast, the crustacean neuroblasts do not delaminate but remain in the apical layer dividing parallel to the surface so that the progenies are pushed to the basal side (Dohle and Scholtz, 1988; Scholtz, 1990; Scholtz, 1992).

It can be speculated that the millipede pattern of neural precursor formation is intermediate when compared with chelicerates and insects (Dove and Stollewerk, 2003). While in the spider neuroectodermal cells seem to divide randomly and are recruited for the neural fate due to their positions in the hemisegment, the presence of neural stem cells in the millipede links cell proliferation and generation of neural precursors in the apical cell layer. Possibly, the necessity to single out epidermal and neural precursor cells from the VNE in insects and crustaceans has led to a separation of the generation sites: epidermal cells are produced in the apical layer, while neural cells are generated on the basal side (Dove and Stollewerk, 2003).

4.4. All cells of the neurogenic region enter the neural pathway in Diplopoda

It is known that after completion of ventral flexure the middle part of each hemisegment sinks into the embryo forming a groove (Dohle, 1964). During the course of neurogenesis this region is gradually incorporated into the embryo, while epidermal cells overgrow the ventral nerve cord. I show here that in *G. marginata* this process does not take place until the final differentiation of the invaginated neural precursors, since a neuropil is already visible on the basal side when the neuromeres sink into the

embryo. Data from *Archispirostreptus sp.* confirms this. Thus, similar to the spider and in contrast to the insects, there is no decision between epidermal and neural fate in the central neurogenic region of the millipede (Stollewerk *et al.*, 2001; Dove and Stollewerk 2003).

4.5. Proneural genes in *G. marginata*

Similar to the spider and also the insects, the *G. marginata achaete-scute* homolog is expressed prior to formation of neural precursors in the VNE. Like the spider *CsASH1* gene, *GmASH* is expressed in patches of cells in the neuroectoderm and becomes restricted to the invaginating cell groups, while in insects proneural gene expression is reduced to one cell of the proneural cluster. After each wave of neural precursor formation, *GmASH* is re-expressed in the regions where invagination sites form, indicating that the gene is necessary for the formation of all neural precursors of the CNS (Dove and Stollewerk, 2003).

In the PNS, *GmASH* is not expressed until stage 4, when transient expression was observed in groups of cells that are assumed to be neural precursors in the head and leg appendages and at the border of the dorsal plates. As the positioning of sensory organs in *G. marginata* has not been studied in detail, it is not possible to say whether these expression domains can be compared to the expression of members of the *D. melanogaster AS-C* in the precursors of the external sensory organs.

GmPannier, like its *D. melanogaster* counterpart, marks the most dorsal part of the embryonic tissue and may well, based on its expression, play an important role in in dorsal closure. While its importance in PNS development is not clear, *GmPannier* expression at the segment borders of the dorsal plates beginning in stage 3 could correlate with the clusters of *GmASH* expressing cells found in these regions beginning in stage 4. Thus is possible that the role of *pannier* in dorsal closure and in the positioning of *achaete* expressing sensory organ precursor cells is conserved between insects and myriapods. Analysis of representatives of the other arthropod groups will reveal if this expression is ancestral.

GmAt, like its *D. melanogaster* homolog, is expressed exclusively in the PNS. Expression in positions where the photoreceptors may develop is transient, while expression in groups of cells in the appendages is constant throughout development. This implies that *G. marginata atonal,* as well as *GmASH*, are necessary for the

development of a subset of sensory organ precursors. However, these assumptions have to be confirmed by functional analysis, as does the hypothesis that the ubiquitously expressed *G. marginata daughterless* homolog plays a similar role to its *D. melanogaster* counterpart as a dimerization partner for bHLH proteins involved in neurogenesis.

4.6. Ancient role for *Sox* factors in neurogenesis

In vertebrates, there are more than 20 *Sox* family members in any given species and most tissues and cell types express a *Sox* gene during at least one stage of their development. Vertebrate Sox proteins are subdivided into eight groups based on their structure, and with the exception of group F, at least one protein from each group is involved in neural development. In particular, vertebrate group B genes (Sox 1, 2 and 3) and SoxD, a distantly related member of the Sox G group, are expressed in a neural-specific manner (Wegner, 1999; Bowles *et al.*, 2000).

In *D. melanogaster*, eight *Sox* genes have been identified, four of which are members of the Sox B family (*Dichaete, SoxNeuro, Sox21a* and *Sox21b*). Two of these genes, *Sox Neuro* and *Dichaete,* have been studied in detail and are expressed widely in overlapping domains in the VNE. *Sox Neuro* mRNA is expressed in the complete VNE but not in the midline, while *Dichaete* is expressed in the most medial part of the VNE and in the midline (Buescher *et al.*, 2002; Cremazy *et al.*, 2000; Overton *et al.*, 2002; Soriano and Russell, 1998). Recent functional studies showed that, in regions where *Dichaete* and *Sox Neuro* overlap, mutant phenotypes are mild compared to the areas where only one gene is expressed, while double mutants show severe neural hypoplasia throughout the CNS. Thus, these two *Sox B* genes have both redundant and differential functions in the CNS, while the function of the remaining *D. melanogaster Sox B* genes has not yet been studied (Overton *et al.*, 2002; Zhao and Skeath, 2002).

I identified three *Sox B* genes in *G. marginata*, and the strong expression pattern of *SoxB1* in the complete VNE, but not in the ventral midline, is strikingly similar to the *D. melanogaster Sox Neuro* expression. Future studies will reveal whether one of the other *Sox B* genes has an expression pattern similar to *Dichaete* and if there are comparable genes in the spider. However, my data appear to support Sasai's theory that *Sox* genes represent conserved neuralizing pathways across species (Sasai, 2001).

Further studies on *Sox* genes in arthropods are, however, necessary to determine to what extent they play a conserved role in establishing these pathways and how this can be compared to *Sox* gene function in other species.

4.7. Neurogenic genes in *G. marginata*

During formation of neural precursors in *D. melanogaster*, the neurogenic genes *Notch* and *Delta* appear to be uniformly expressed in the neuroectoderm (Baker, 2000). Although it is assumed that, within a proneural cluster the cell expressing the highest amounts of *Delta* is selected for the neural fate, no variation in *Delta* expression has yet been observed in the VNE of fly embryos. In contrast, the expression patterns of the spider *Delta* genes can be correlated to the formation of neural precursors. While *CsDelta1* is exclusively expressed in neural precursors, *CsDelta2* transcripts are distributed uniformly throughout the neuroectoderm and accumulate in nascent neurons (Stollewerk, 2002).

The only *Delta* gene I found in *G. marginata* is expressed similar to the spider *CsDelta2* gene: at low levels in almost all neuroectodermal cells and at higher levels in the invaginating cell groups. Furthermore, although *G. marginata Notch* is initially expressed uniformly in the neuroectoderm, it resolves into a heterogenous expression during the first wave of neural precursor formation similar to the spider *CsNotch* transcripts. Thus the expression patterns of the neurogenic genes in *G. marginata* and *C. salei* are more similar to each other than to the expression of their *D. melanogaster* homologs (Dove and Stollewerk, 2003).

4.8. Dorso-ventral patterning in the ventral neuroectoderm

The expression of *GmInd* and *GmMsh* in medial and lateral domains, respectively, can be compared to the situation in *D. melanogaster*, where homologs specify medial and lateral columns of neuroblasts. Comparable to the first wave of neuroblast formation in *D. melanogaster* embryos, *GmMsh* RNA is visible in a lateral column of cells with heterogenous expression levels in each hemisegment; however, while expression is restricted to specific lateral neuroblasts in later stages of *D. melanogaster* development,

no restriction of *GmMsh* to invagination sites could be observed in this region (Isshiki *et al.*, 1997).

Additionally, and in contrast to *D. melanogaster, GmMsh* is expressed in a small medial domain in stage 5 embryos, which correlates to the region in which the last invagination sites have formed. It is possible that the medial *GmMsh* is restricted to specific invagination sites.

In consideration of these two final points, it will be interesting to see whether the expression pattern of spider *msh* is more comparable to *G. marginata* or to *D. melanogaster* and what the significance of the medial *GmMsh* expression is.

GmInd expression is similar to the expression of its *D. melanogaster* homolog in that it is rapidly restricted to a small intermediate cluster of cells. After the initial expression in an intermediate column of cells in each hemisegment, *D. melanogaster ind* is restricted to one neuroblast by the end of the third wave of neuroblast formation (Weiss *et al.*, 1998). To see if the group of cells expressing *GmInd* actually forms a single invagination site, double staining with a neural antibody or an in-situ hybridization with a probe such as *prospero* or *snail,* which has a panneural pattern of expression, could be performed in future. It will be interesting to isolate a *vnd* homolog from *G. marginata* in order to study its expression pattern, and to see whether there is an overlap of *msh* and *vnd* in the medial *msh* expression domain.

The conservation of expression of *msh* and *ind* homologs in lateral and intermediate positions in the VNE of *G. marginata* in itself is not surprising, since vertebrate homologs *msx* (*msh*), *gsh* (*ind*), and *Nk2.1* (*vnd*) are expressed in dorsal, intermediate and ventral domains of the dorsal neuroectoderm during spinal cord development, comparable to the expression of their *D. melanogaster* homologs in the VNE. Thus, dorso-ventral patterning via *msh*, *ind* and *vnd* homologs represents an ancient positioning system (Arendt and Nübler-Jung, 1999 and references therein). However, identification of invagination sites that arise within these domains will allow for the comparison of subsets of neural precursors and their progeny between spiders and millipedes. Since *G. marginata ind* and *msh* homologs are expressed in domains as are their counterparts in vertebrates - rather than in single cells, as in the case for the *D. melanogaster* homologs - their expression patterns are more similar to vertebrates than to insects.

4.9. Cell fate specification and neural markers

In the *D. melanogaster* neuroectoderm, the *snail* genes are necessary for the asymmetric distribution of the cell fate determinants Prospero and Numb to ganglion mother cells. Only one *snail* gene was identified in *G. marginata,* which, like its *D. melanogaster* and spider homologs, is specifically expressed in most neuronal precursor cells of the CNS as soon as they have formed. While *GmSnail* expression was observed in all invagination sites, it is not clear if all cells of an invagination site express *snail*. Additionally, beginning at stage 2, *GmSnail* shows strong expression in the ventral midline, which can be compared to the mesodermal expression in *D. melanogaster*; however, this expression has not been observed in the spider *Cupiennius salei*. Unlike the *D. melanogaster* and *C. salei snail* homologs, *GmSnail* is not expressed in the PNS, at least up to stage 5 of development.

Both *snail* and *prospero* expression is visible in all invagination sites of the *G. marginata* VNE, but while levels of *snail* appear homogenous in all invagination sites, *prospero* is expressed more strongly in the most medial neural precursors and is present in a subset of precursors of the PNS. Despite the panneural expression mode of both genes, it is not clear whether the presence of *snail* and *prospero* in the *G. marginata* VNE indicates that they are involved in cell fate specification via asymmetric cell division. In the spider *C. salei*, antibody staining did not reveal asymmetric localization of Prospero or Snail in the CNS, and no asymmetric localization has been reported for vertebrate Prox1 (Weller, 2002; Torii *et al.*, 1999). In addition, in the *D. melanogaster* PNS, Prospero plays a role in neuron differentiation, but no asymmetric distribution has been reported (Cai *et al.*, 2001).

Snail and *prospero* expression in neural precursors, as well as ubiquitous *numb* expression, may be ancestral to arthropods. However, without studies of protein distribution and functional data nothing certain can be said about the importance of these genes in *G. marginata*. At present, due to their panneural expression, *prospero* and *snail* can be considered markers of neural precursors and their expression in the CNS is comparable to late *GmDelta* expression in all invagination sites.

GmIslet was isolated in an attempt to identify genetic markers for subsets of neural progeny. However, it was found that while *GmIslet* is highly homologous to the *Cupiennius salei* gene, in contrast to the spider, no clear expression in subsets of neurons could be detected (Stollewerk, unpublished data). It is possible that expression

in subsets of neurons is present in later stages of neurogenesis when the *G. marginata* embryos have formed a waxy cuticle making studies of gene expression difficult. Comparison of the expression of other genes that mark subsets of neurons or neural precursors in insects, such as *engrailed*, *even-skipped* or *seven-up*, between *C. salei* and *G. marginata* will show whether specific invagination sites and neurons can be identified by the genetic markers they express.

4.10. Phylogenetic positioning of Myriapoda

My data support the hypothesis that myriapods are closer to chelicerates than to insects. The spider and the millipede share several features that cannot be found in equivalent form in the insects: in both the spider and the millipede, about 30 groups of neural precursors invaginate from the neuroectoderm in a strikingly similar pattern. Furthermore, in contrast to the insects, there is no decision between epidermal and neural fate in the VNE of both species analysed (Dove and Stollewerk, 2003). The sequence data, so far as it can be compared at this time, also suggest a closer relationship of the millipede to the spider as opposed to the insects. However, to confirm a sister group relationship of these arthropod groups, more morphological data from different representatives of myriapods, chelicerates and outgroups will be necessary.

4.11. A universal genetic network for nervous system development?

Based on the comparison of my data to what is known from insects and the spider as a representative of the chelicerata, it can be postulated that there is a conserved genetic network that controls neural development within Arthropoda. Neural competence is conferred to groups of cells in the VNE by the proneural genes and members of the *Sox* family. The number of cells destined to become neural precursors at any one time is restricted by the Notch signalling pathway. Patterning of the neuroectoderm occurs through a conserved set of transcription factors that are expressed in clear spatio-temporal patterns, which confer individual identity to each neural precursor. Of course, this is only a hypothesis and further data from different arthropods, especially further molecular data from crustaceans and chelicerates as well as from other myriapods and

outgroups, will help define a general mode of neurogenesis for arthropods, which can then be directly compared to what is known from other clades.

The genetic network controlling arthropod neurogenesis is also necessary for neural development in vertebrates, making it conceivable that the last common ancestor of vertebrates and invertebrates had a much more sophisticated nervous system than has been assumed until now. This supports the theory, presented by Arendt and Nübler-Jung, that the CNS of arthropods and vertebrates did not evolve independently (Arendt and Nübler-Jung, 1999). It is surprising that the genetic network controlling neural development is conserved between arthropods and vertebrates, despite the fact that the morphological aspects of neurogenesis are very different.

Recent data from the hemichordate *Saccoglossus kowalevskii,* shows that, even though this species only has a simple nerve net, gene expression in specific domains in the nervous system is conserved (Lowe *et al*., 2003). This conservation of the genetic network specifying neurogenesis supports the hypothesis that this network is, indeed, ancestral to arthropods and vertebrates.

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6. Appendix

6.1. Sequences

Sequences are listed in alphabetical order. The putative amino acid sequences are displayed below the open reading frame.

***Achaete-scute homolog*, Accession number AJ36345**
Archispirostreptus species

The conserved domains at the beginning and the end of the putative amino acid are underlined, as is the basic-helix-loop-helix domain which is also in bold type.

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                    10        20        30        40        50        60
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH   1 ACACTGACATGGACTGAAGGAGTAGAAAACACACCGATACATCCAGCCGACAGATAAACA
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH  61 CGCGGCTCGGAGTAGGGCATACCAGAACTTGTTATAAACACTTCGGACTTAGAGCATCCG
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 121 TCTGAGTGAAAAGTGATTTTTGATTTTTCGGTCGTTTTTTATTTTTATTTATTATTGCTA
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 181 TTATTATTATTCATTTTTATATATTTATATACAACTACCCGAGACTCTGACATTGGTTTA
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 241 CCGGTATAATTCGTGAACCATCGACTCTTTTTTTTTATTTTTTGAAAAAAATTTTTTTTG
                                                    *  K  K  F  F  L
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 301 CACGGACAGAGTTATTCGCTCCTAATAACGCGAGACATGGCCGTGTCAACCACCATTCTG
           H  G  Q  S  Y  S  L  L  I  T  R  D  M  A  V  S  T  T  I  L
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 361 CATCCCACTCTACAGAACTCCGTTCACTTAAATCTGAGCCCCGACAAACGGACCGTTTCG
           H  P  T  L  Q  N  S  V  H  L  N  L  S  P  D  K  R  T  V  S
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 421 AGCGGCAACGACAACGACGGTGCGTACGTGTACTTAAACGCGTCGACGACGGTACCCAAC
           S  G  N  D  N  D  G  A  Y  V  Y  L  N  A  S  T  T  V  P  N
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 481 CTGCACCCGGCCGCCGTGCCCAGTTCGACGACGACGACGACGACGGTCGCGAGGAACCCG
           L  H  P  A  A  V  P  S  S  T  T  T  T  T  T  V  A  R  N  P
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 541 TCGGTCACCGTCGGCAACCGAATCCTCGGCAGCGGCGCAGCGACCACCGTGAAAATGAGC
           S  V  T  V  G  N  R  I  L  G  S  G  A  A  T  T  V  K  M  S
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 601 GTGAAGCGAGCGCGAGGCAGCGGCAGCGAGAGCTGTGGCAGCATCACGCCGCCCGAGATG
           V  K  R  A  R  G  S  G  S  E  S  C  G  S  I  T  P  P  E  M
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 661 ATGCGGTGCAAGCGGCGGATCAACTTCGCGCAGCTCGGATACAACCTTCCACAGGCGCAG
           M  R  C  K  R  R  I  N  F  A  Q  L  G  Y  N  L  P  Q  A  Q
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 721 CCTTCGGCCGTNTCCAGGCGCAACGAACGCGAGCGCAACCGAGTCAAGCTGGTCAACCTG
           P  S  A  X  S  R  R  N  E  R  E  R  N  R  V  K  L  V  N  L
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 781 GGCTTCGCCACGCTACGCGAGCACGTGCCCAACGGCAGCAAGAACAAGAAGATGAGCAAA
           G  F  A  T  L  R  E  H  V  P  N  G  S  K  N  K  K  M  S  K
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 841 GTGGAGACGCTGCGCTCGGCCGTTGAGTACATCAGAAAGTTGCAGGAGCTGCTCGATGAG
           V  E  T  L  R  S  A  V  E  Y  I  R  K  L  Q  E  L  L  D  E
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 901 ACGGAGGCCGGCGCGAGCAGCCCGTACGCGGACTCTTCCTCGTCCATGGCCGGTTCGCCG
           T  E  A  G  A  S  S  P  Y  A  D  S  S  S  S  M  A  G  S  P
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 961 GTGCAAGCTGAGAACTTCATTCCGAACGGCGTGGTCGTCGGCGTCGGCAGCAGCAACGGT
           V  Q  A  E  N  F  I  P  N  G  V  V  V  G  V  G  S  S  N  G
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH1021 GCCGCGGACGGCGCCTTATTTCCCCGCAGCGGACTATCGTGCCCTCCGTCCAGCGTCTCG
           A  A  D  G  A  L  F  P  R  S  G  L  S  C  P  P  S  S  V  S
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH1081 CCGACGTGTTCGGTGGCCAGTTCGCCGACGCCCAGCTACACGTCGGACTATTCTTACGAA
           P  T  C  S  V  A  S  S  P  T  P  S  Y  T  S  D  Y  S  Y  E
          ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH1141 CCCCTCAGTCCCGAAGAGGAAGATCTCCTAGATTTCACAAGCTGGTTCTCTTAATGAACA
           P  L  S  P  E  E  E  D  L  L  D  F  T  S  W  F  S  *  *
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            ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 1201 GAGTTTCGCCCGCCTTCTTTAACGGTAAGTCGGAAATTTTATAATTAGCGCAACGCGTTT
            ....|....|....|....|....|....|....|....|....|....|....|....|
AsASH 1261 CGGTAGCGAATCGATTCCCCCCTTATTAGAACCGAACGTCCCTGGACGTCACACACGTTA
1320
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***Achaete-scute homolog*, Accession number AJ36346**
Glomeris marginata

The conserved domains at the beginning and the end of the putative amino acid are underlined, as is the basic-helix-loop-helix domain which is also in bold type.

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  1    GGACACTGACATGGACTGAAGGAGTAAAAAAGCATTCGCTTTGGCGAAGTCTCGCAGAGA
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  61   GGAGCTAGCTGCAACTGCACACTGCAACCACCAGCTGCTGCTGCTTGCGCACATCTCACA
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  121  TTTTCTGCTTGAACTGAGCAGATACCACCAGCACGTGGCTTCATCTTACACTTGGACTGA
                                                                        *
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  181  ACGAATAGAGCGAGAAGCACGCGCCAGGCACACGCCATGGCCACCACTGCTAATATGAGT
             T  N  R  A  R  S  T  R  Q  A  H  A  M  A  T  T  A  N  M  S
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  241  GCAGATCAAGGTTTCTTGTATGTTAAGAGCGAGAATTCCGAGCACAGTCACAACCACCAC
             A  D  Q  G  F  L  Y  V  K  S  E  N  S  E  H  S  H  N  H  H
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  301  CACCAACACCATGGTCACAAAAGCAGCCACAAGCGGCCCAGGATCGAACCTTTACCTGCT
             H  Q  H  H  G  H  K  S  S  H  K  R  P  R  I  E  P  L  P  A
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  361  GCAGCTACAAACGATGGCAATTCATCTTCTTCATCTTCCTCCGAATCACCTTCAACTCCG
             A  A  T  N  D  G  N  S  S  S  S  S  S  S  E  S  P  S  T  P
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  421  GAATTGATGCGTTGCAAGAGAAGGATCAACTTTGCACAGATGGGCTACAATCTTCCTCAA
             E  L  M  R  C  K  R  R  I  N  F  A  Q  M  G  Y  N  L  P  Q
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  481  GCTCCACCAGCAGCAGTTGCTCGGAGGAACGAAAGGGAACGAAACAGGGTCAAGTTGGTC
             A  P  P  A  A  V  A  R  R  N  E  R  E  R  N  R  V  K  L  V
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  541  AACATGGGTTTCGCAACACTCAGGGAACACGTCCCAAACGGCGTCAAGAACAAGAAGATG
             N  M  G  F  A  T  L  R  E  H  V  P  N  G  V  K  N  K  K  M
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  601  AGCAAAGTGGAGACTCTGCGTTCGGCAGTGGACTACATAAAAAGACTCCAAGAGCTGCTA
             S  K  V  E  T  L  R  S  A  V  D  Y  I  K  R  L  Q  E  L  L
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  661  GATGAGCAGAACCCTGGTGGTTCGAGTTCTGGGTCTGCTGCGGCTGCTGCTGCAGCAGCT
             D  E  Q  N  P  G  G  S  S  S  G  S  A  A  A  A  A  A  A  A
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  721  CTGGCAGGACTGGCAGATACTTTCGCGTCCGACGGTAGTACCACTGGCGACGGGGACGGT
             L  A  G  L  A  D  T  F  A  S  D  G  S  T  T  G  D  G  D  G
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  781  TGTTACAATAACGGTTCAGGAGGTGGTGGAGATGCTGGACGTGTTAACGGTGATGGTGCT
             C  Y  N  N  G  S  G  G  G  G  D  A  G  R  V  N  G  D  G  A
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  841  AGTACTGGTAATAATGGGTCTGCAACAGGGGGTAACATTTGCTTGCCATCTAGCGTGTCA
             S  T  G  N  N  G  S  A  T  G  G  N  I  C  L  P  S  S  V  S
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GmASH  901  CCCACCGTGTTCTGTTGCAAGTTCCCCAACTCCGAGTTACGCTTCAGACTGTTCCTCGTC
             P  T  V  F  C  C  K  F  P  N  S  E  L  R  F  R  L  F  L  V
                    ....|....|....|....|....|....|....|....|....|....|....|..
GmASH  961  GAACCTTTGAGTCCAGAAGAAGAGGATNTGCTCGATTTCACCACGTGGTTCTCCTAA 1017
             E  P  L  S  P  E  E  E  D  X  L  D  F  T  T  W  F  S  *
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***Achaete-scute homolog*, Accession number AJ36347**
Tribolium castaneum

Only the conserved basic-helix-loop-helix domain was amplified by RT-PCR. The fragment shown here was used for phylogenetic analysis.

```
                10        20        30        40        50        60
           ....|....|....|....|....|....|....|....|....|....|....|....|
Tc ASH 1   AGAGAGCGGAATCGTGTCAAGGTCAATAATGGGTTTGCCAATTTGCGCCAACACATCCCG
            R  E  R  N  R  V  K  V  N  N  G  F  A  N  L  R  Q  H  I  P
           ....|....|....|....|....|....|....|....|....|....|....|....|
Tc ASH  61 AATTTCATCGCAGCCGCTTTCGAGTCCAACAGTCGAGGGGGCAACAAGAAACTCAAAGTA
            N  F  I  A  A  A  F  E  S  N  S  R  G  G  N  K  K  L  K  V
```

Atonal homolog
Glomeris marginata

The conserved basic helix-loop-helix domain is bold and underlined.

```
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  1     GCTTTTGATAAACTCCGAGATGTGGTACCATCACTTGGAAACGACAGGAAATTGTCAAAG
             A  F  D  K  L  R  D  V  V  P  S  L  G  N  D  R  K  L  S  K
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  61    TACGAGACTTTGCAAATGGCGCAGACTTACATATCCGCATTACTTGAACTCCTTCACCGA
             Y  E  T  L  Q  M  A  Q  T  Y  I  S  A  L  L  E  L  L  H  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  121   GACTGAACTGAAGAGCAACATTTGCTTCAAATATATATTATATGAGTACTGTATATAGTT
              D  *
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  181   GCGTTGTGTATATAGACCACATAATTTTCGAACAGTGGGTTCAACTTTTGAAGATTATGG
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  241   TCTAAGTGTACAGAAACACTTTTGTATGAGAGAGAGAGAGAGGGAGAGCTGGGATTTTTG
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  301   CATCCACAGATCGATCTCAGGTCGTAGATATACTTTTTCTTTACAAAAGTTAATTTCCAA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  361   TATGTAAGATAGAAAATATAAGGTTGCCACAACTGTTAATATTCAATTTCTAAATTATCC
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  421   TCAAAAAATGACTCGACTGCATTCTGAATTCGTGGCTCTGAGAAAAATACAAAACACGGA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  481   ATCATTGCACACAAAATCAATATAAATGTTTCGTTATTCCTTGGAAAATAGCACTCCATA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  541   AATAATAACAATAAAAAACTATTCAGATCAATGCAGGGCTGCGTACATCAGAAATGTGGT
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  601   TTTGTAATAATTCATTTTCGTAAAAATGTTAGTTGAATCGTTTTTCAAGATTGTGCTTCT
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  661   TCGATTGTATGAAATAAATTTAACAGTTCTGAAAATCGTTAAAATATTCAACCATGATTA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  721   TATTTCTTGAATAGTAGATGTTTTGTATGAATTCCTTAATTTAGACATTTTCTCGTAATT
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  781   CTGATTTTACAAATTTTCATTATTCGATCTACTTAATTGCTGTTCATGTGACTAAAAATT
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  841   AAGTTAACAATTAACATTTATAAAATCAACGACTTTCACATCATCAGATAAATTCCTATA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  901   GTATGCATTATACTAATTTTTGAAACAAATTCCATTAATATCCATAATATTTCTTAAACA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  961   TTCCCTGATTTACAAATACATATATATTCGGTGATGTTTTATAATTTTTTGGCATCTCCA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  1021  TGTATTAATATGATAGAAAACTATTCGTATTTCATTACCTTTCCATTTGAGTGGCAACCT
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  1081  TATAAAAGAAGTACTATAAAAGGTGGTAAAATGTGATAATTGGCACGATCAATTTATAGC
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  1141  TCACTGAAAATGCTCAAACGATTGCCAAGAAAAGCGAATGAAAACGTTTTAAAGTTAACA
                ....|....|....|....|....|....|....|....|....|....|....|....|
GmAt  1201  AACGCTAAAAAGAGTGTTTCTTTATATCATGACTTGATGAAAATGTTTCAATCAGGATTT
                ....|....|....|....|....|....|....|.
GmAt  1261  CAATTTATTTTAATTGTCCTGCATGAATATGTGTATAAAGC 1301
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***Daughterless* homologs**
Glomeris marginata

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                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da       1   ATACGCGATATTAATGAGGCCTTCAAAGAACTCGGCAGAATGTGCATGGTGCATCTGAAA
                 I  R  D  I  N  E  A  F  K  E  L  G  R  M  C  M  V  H  L  K
Gm da 2     1   --T--G--C--C--C-----T-----G--G--G---C-C---GTTCA-C----C-----G
                 I  R  D  I  N  E  A  F  K  E  L  G  R  M  V  Q  L  H  L  K

                    70        80        90       100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da      61   ACAGATAAAGCACAGACGAAATTAAACATACTTCATCAGGCAGTGGAAGTGATTACCAGT
                 T  D  K  A  Q  T  K  L  N  I  L  H  Q  A  V  E  V  I  T  S
Gm da 2    61   -GC--A--GC-C-----C--GC-GCT---C--G--C-----C----C---C--ACTG--C
                 S  E  K  P  Q  T  K  L  L  I  L  H  Q  A  V  A  V  I  L  S

                    130       140
                ....|....|....|....|...
Gm da      121  TTAGAGCAACAAGTCAGCGAGCG
                 L  E  Q  Q  V  S  E
Gm da 2    121  C-G--A-----G---C-----A-
                 L  E  Q  Q  V  R  E
```

***Daughterless* RACE fragment**
Glomeris marginata

Conserved domains are bold and underlined.

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                    10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   1   ACCAGCGACGACGAACCGCTTCATCTCTATGAAGTTTTCCAGAACTGCTTCAATAAGATA
              T  S  D  D  E  P  L  H  L  Y  E  V  F  Q  N  C  F  N  K  I
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   61  ACTAACAGGCAGCCAGATAAAAGCAGTTATCCAATGCCGTATTCCACGATGCAAGCCGAC
              T  N  R  Q  P  D  K  S  S  Y  P  M  P  Y  S  T  M  Q  A  D
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   121 AACGGCATGTCTCAGGCTTATCCAAACTCGTTCAGTGGTGCAACGGAATCATCGTTGACT
              N  G  M  S  Q  A  Y  P  N  S  F  S  G  A  T  E  S  S  L  T
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   181 CCTGACTCTCCGTACTTTCCTTTCAGCAATGGTGCTCAACGTCGAGCATTTCCTCATGGT
              P  D  S  P  Y  F  P  F  S  N  G  A  Q  R  R  A  F  P  H  G
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   241 GCCTCAGCAAATAAACGGAAAAGAGATGTTGTGGAATCAGAAAACATGAACCTGCCATGG
              A  S  A  N  K  R  K  R  D  V  V  E  S  E  N  M  N  L  P  W
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   301 TATGGTTCCGAAGAGTTCGGACAAGACTCGCCTCGGTATACCTCTCCGAAACCGGGCCTT
              Y  G  S  E  E  F  G  Q  D  S  P  R  Y  T  S  P  K  P  G  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   361 TACGGAGAATCCTACTTTGTGGGCGGTGAAGGCGCTCACAACGCCAATGACCCGTGGTCA
              Y  G  E  S  Y  F  V  G  G  E  G  A  H  N  A  N  D  P  W  S
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   421 TCTAGCAACAATCTACCATCATCCAGTTACTCATATTCGTCGTCCATGATGGGTACAGGG
              S  S  N  N  L  P  S  S  S  Y  S  Y  S  S  S  M  M  G  T  G
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   481 TCTCACCTCACACAGCCCTCAGCCGCCTACCCCAGTATGCATCTTCCACATGACACTATG
              S  H  L  T  Q  P  S  A  A  Y  P  S  M  H  L  P  H  D  T  M
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   541 GGTTACCATACGATGTCACCGAACCACGATCCCAGCATGATGGCCACNAGTCTTCCACCC
              G  Y  H  T  M  S  P  N  H  D  P  S  M  M  A  X  S  L  P  P
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   601 ATGTCCAGCTTTCGAGGGACAGGGACCCCGGGTCTGCAACCGACAGCAGCCATCACGCAC
              M  S  S  F  R  G  T  G  T  P  G  L  Q  P  T  A  A  I  T  H
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   661 TCTTCTTCCTCGCCGCTTTACGCCAACGCCAACCCATCACCGACAATAAGTACAGCCGAA
              S  S  S  S  P  L  Y  A  N  A  N  P  S  P  T  I  S  T  A  E
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   721 TCCCTCATCAGTAGGCAGGATCAGAGCGCATCGCAAACCGGTGATGCACTCAGCAAAGCC
              S  L  I  S  R  Q  D  Q  S  A  S  Q  T  G  D  A  L  S  K  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   781 ATTGCATCTATGTATTCTGCGGATCATACGAGTAGCAGCTTCTCTTCGAATCCCTCCACG
              I  A  S  M  Y  S  A  D  H  T  S  S  S  F  S  S  N  P  S  T
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   841 CCTGTCAGCTCACCGCCACCCATATCTGGTCGTACGCAGTGGACCTCTCACGGGCCATCG
              P  V  S  S  P  P  P  I  S  G  R  T  Q  W  T  S  H  G  P  S
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   901 CAGACCGGGCCGGCGTCTCCGCACCTTGTAGATGGACGTCTTCGGTCACTTCAACAGACA
              Q  T  G  P  A  S  P  H  L  V  D  G  R  L  R  S  L  Q  Q  T
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da   961 CATATCGAAGAACGGTTGGACGATGCCATCAACGTGCTGCGAAACCACGCCGAAGGTGCA
              H  I  E  E  R  L  D  D  A  I  N  V  L  R  N  H  A  E  G  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da  1021 TCGCTGCACAGTTTACCGGGTGCTCCCAGCTCTGCGGTGGGGACCTCGGGAACAAGTTTG
              S  L  H  S  L  P  G  A  P  S  S  A  V  G  T  S  G  T  S  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da  1081 CCTGTTCACTCGGGGACGCCACATTCCAACGGCCTCATTGGCACTAGCATCAGTTCATCA
              P  V  H  S  G  T  P  H  S  N  G  L  I  G  T  S  I  S  S  S
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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1141      TTCCCAGCCAGTGCGGGAATGTCTCCCATAGAAGCACATTTGCCAAGCCCACATGGAATA
                 F  P  A  S  A  G  M  S  P  I  E  A  H  L  P  S  P  H  G  I
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1201      TCTGACAGTAGATTAAGGAATAGGGAAAGTGGAGGAGTGCCAACAGCTAGTGGATTGTCT
                 S  D  S  R  L  R  N  R  E  S  G  G  V  P  T  A  S  G  L  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1261      GGCCAATCAGATCAGCACACAGATTCGAATGGTGGGCTGAAGGTTGACCGTCTCTCCGAG
                 G  Q  S  D  Q  H  T  D  S  N  G  G  L  K  V  D  R  L  S  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1321      AAGAGGAAAGAACCAGATGGCAGCAAGTCTCCGCCACCCGGAGCAACATCTTCGGTGCCG
                 K  R  K  E  P  D  G  S  K  S  P  P  P  G  A  T  S  S  V  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1381      TCGTCGGGTCCTGGGTCTGTTCCATCCGGTGCACCAAATAGTGTTAGCACAACAACGTCA
                 S  S  G  P  G  S  V  P  S  G  A  P  N  S  V  S  T  T  T  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1441      TCTCATCACAAAGGCGCCAAAAGAGCCCGTTCTAGTTCTCCGCTGCTGCACGGAGACGAA
                 S  H  H  K  G  A  K  R  A  R  S  S  S  P  L  L  H  G  D  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1501      GACGAGTCTCCGGAGCCGAAAGCCGAAAGGGAGAAAGAAAGGAGGCAGGCAAACAATGCA
                 D  E  S  P  E  P  K  A  E  R  E  K  E  R  R  Q  A  N  N  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1561      CGAGAAAGGATCCGTGTGCGTGATATAAATGAGGCCTTCAAAGAACTCGGCAGAATGTGC
                 R  E  R  I  R  V  R  D  I  N  E  A  F  K  E  L  G  R  M  C
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm da 1621      ATGGTGCATCTGAAAACAGATAAAGCACAGACGAAATTAAACATACTTCATCAGGCAGTG
                 M  V  H  L  K  T  D  K  A  Q  T  K  L  N  I  L  H  Q  A  V
                                        bHLH domain
                ....|....|....|....|....
Gm da 1681      GAAGTGATTACCAGTTTAGAGCAACAAGT 1709
                 E  V  I  T  S  L  E  Q  Q
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***Delta homolog*, Accession number AJ36343**
Archispirostreptus species

The DSL domain is marked in bold and underlined, followed by EGF repeats 1 and 2, which are in italics and underlined.

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                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 1     ATCGTGGAAGCGTGGCACGAACCTATCCAGGCTGCTTCCTCTGGAGGAGCGCCATCTTCT
               I  V  E  A  W  H  E  P  I  Q  A  A  S  S  G  G  A  P  S  S
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 61    CCAACGTTAATCACGCGGTTAACAACGCAAGGCTGGCTAGCTGTGGGATTAAATTGGACA
               P  T  L  I  T  R  L  T  T  Q  G  W  L  A  V  G  L  N  W  T
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 121   AATGCCACACACCAAAGTGGAAATATTACGCTGCATTACGCGTATCGCGTACATTGCAGC
               N  A  T  H  Q  S  G  N  I  T  L  H  Y  A  Y (R  V  H  C  S
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 181   GTTAATTATTACGGTAACATTTGTGGGGATTTGTGTCGCCCTCGTGACGACAAGTTCGGC
              V  N  Y  Y  G  N  I  C  G  D  L  C  R  P  R  D  D  K  F  G
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 241   CACTTCAACTGTTCGCCTACGGGGGCGAAAGTGTGCCTTCCAGGTTGGACTGGCGAATAC
              H  F  N  C  S  P  T  G  A  K  V  C  L  P  G  W  T  G  E  Y
                                          DSL
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 301   TGCTCCAAAGCTGTTTGCGCCCCTGGCTGTCACGAAGAACATGGCGGCTGCGATGCCCCC
              C)(S  K  A  V  C  A  P  G  C  H  E  E  H  G  G  C  D  A  P
                                        EGF 1
                    ....|....|....|....|....|....|....|....|....|....|....|....|
AsDelta 361   AACGAATGCAAGTGTCGGATGGGTTGGCAGGGCAAGTTGTGTGACCAGTGCATTCGGTAC
               N  E  C  K  C  R  M  G  W  Q  G  K  L)(C  D  Q  C  I  R  Y
                    ....|....|....|....|....|....|....|....|....|....|...
AsDelta 421   CCTGGCTGCCTTCAGGGTACGTGCAACAGTCCGTGGCAGTGCAACTGCGACGA 473
               P  G  C  L  Q  G  T  C  N  S  P  W  Q  C  N  C  D
                                        EGF 2
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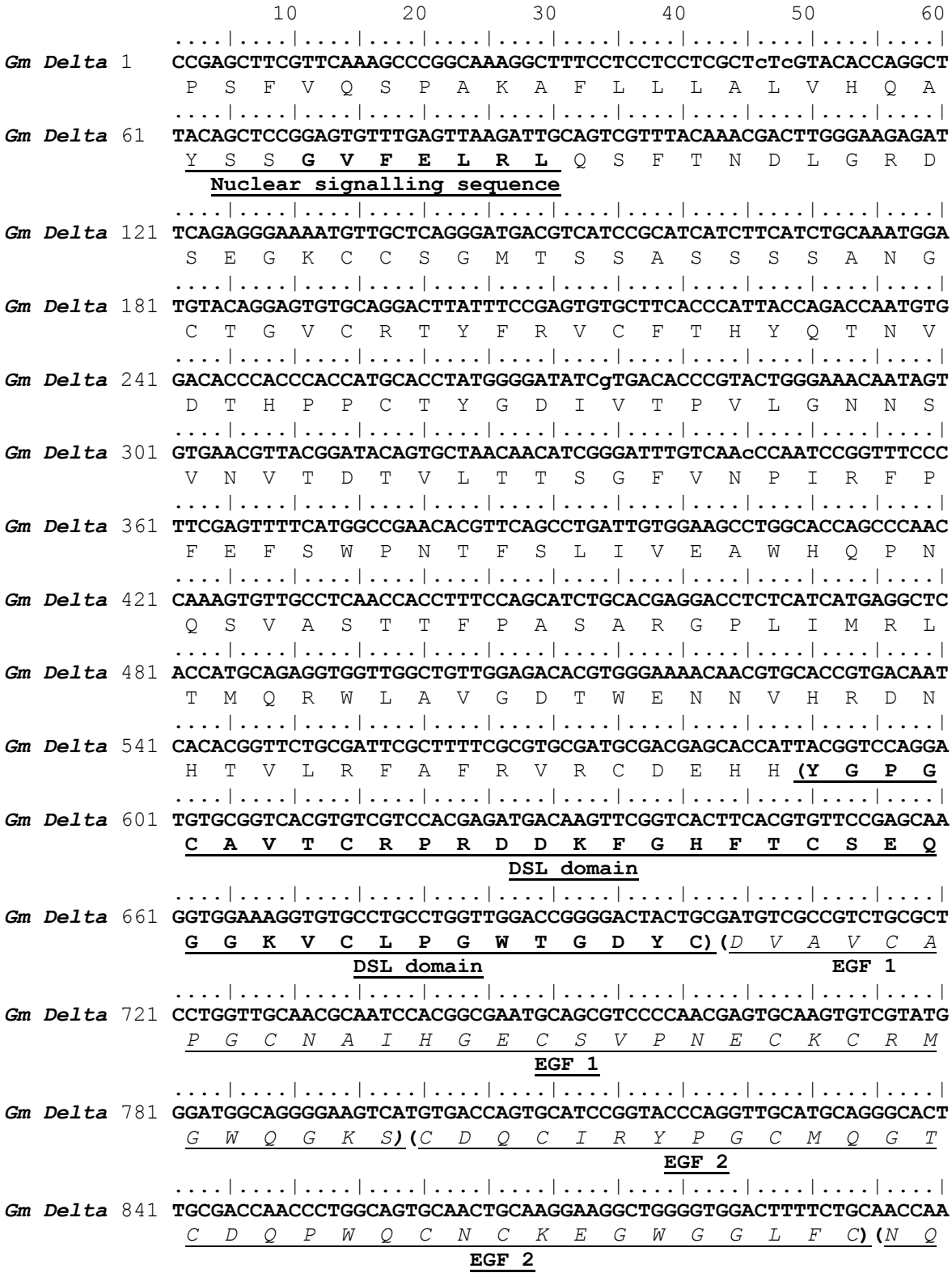
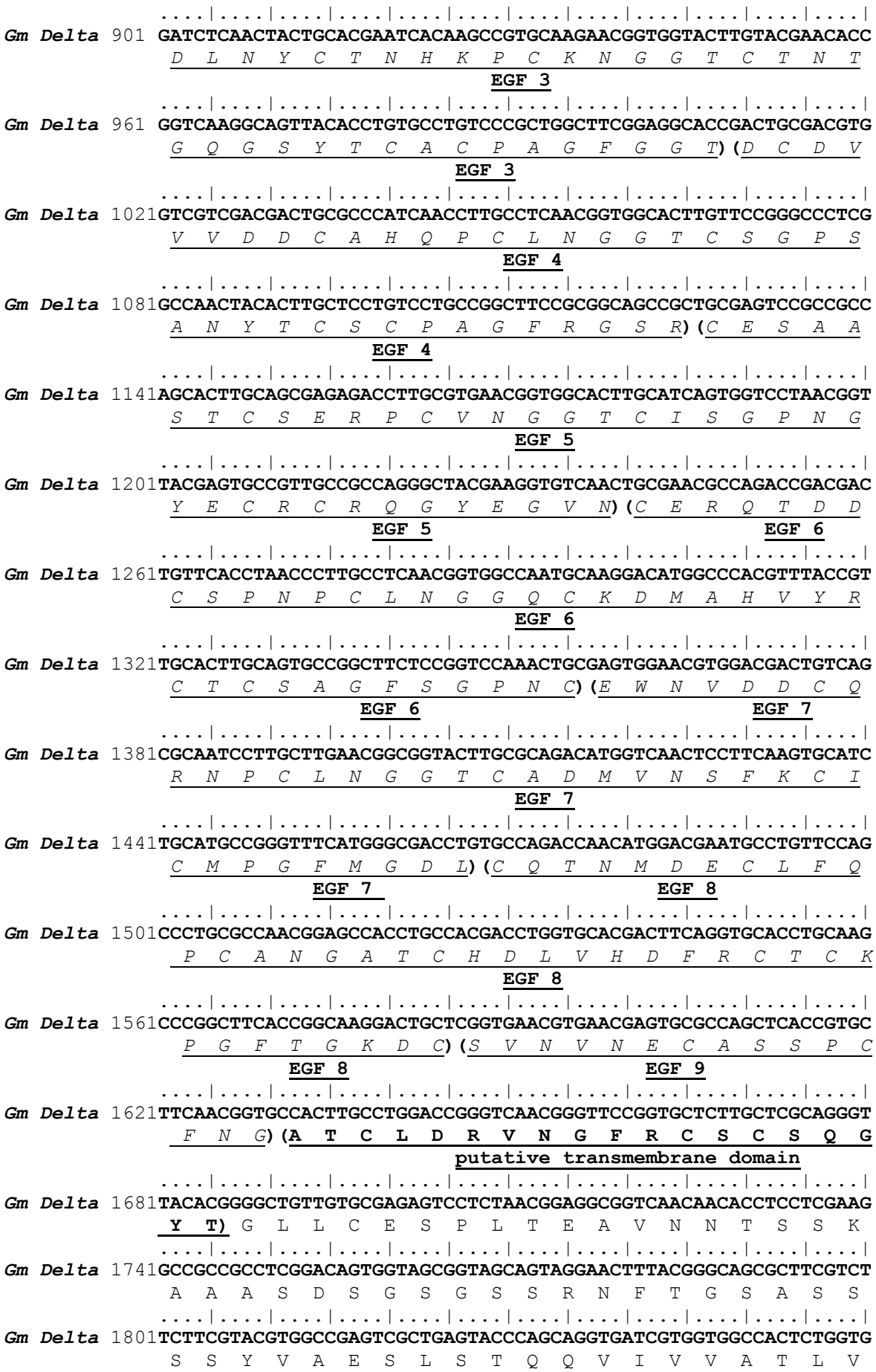
***Delta* homolog, Accession number AJ36341**
Glomeris marginata

The conserved nuclear signaling sequence is followed by the Delta-Serrate-Lag domain (DSL), both marked in bold and underlined. The EGF repeats 1-9 are underlined and in italics, and the putative transmembrane domain is bold and underlined.




Gm Delta 901 GATCTCAACTACTGCACGAATCACAAGCCGTGCAAGAACGGTGGTACTTGTACGAACACC
D L N Y C T N H K P C K N G G T C T N T
EGF 3
Gm Delta 961 GGTCAAGGCAGTTACACCTGTGCCTGTCCCGCTGGCTTCGGAGGCACCGACTGCGACGTG
G Q G S Y T C A C P A G F G G T) (D C D V
EGF 3
Gm Delta 1021GTCGTCGACGACTGCGCCCATCAACCTTGCCTCAACGGTGGCACTTGTTCCGGGCCCTCG
V V D D C A H Q P C L N G G T C S G P S
EGF 4
Gm Delta 1081GCCAACTACACTTGCTCCTGTCCTGCCGGCTTCCGCGGCAGCCGCTGCGAGTCCGCCGCC
A N Y T C S C P A G F R G S R) (C E S A A
EGF 4
Gm Delta 1141AGCACTTGCAGCGAGAGACCTTGCGTGAACGGTGGCACTTGCATCAGTGGTCCTAACGGT
S T C S E R P C V N G G T C I S G P N G
EGF 5
Gm Delta 1201TACGAGTGCCGTTGCCGCCAGGGCTACGAAGGTGTCAACTGCGAACGCCAGACCGACGAC
Y E C R C R Q G Y E G V N) (C E R Q T D D
EGF 5 EGF 6
Gm Delta 1261TGTTCACCTAACCCTTGCCTCAACGGTGGCCAATGCAAGGACATGGCCCACGTTTACCGT
C S P N P C L N G G Q C K D M A H V Y R
EGF 6
Gm Delta 1321TGCACTTGCAGTGCCGGCTTCTCCGGTCCAAACTGCGAGTGGAACGTGGACGACTGTCAG
C T C S A G F S G P N C) (E W N V D D C Q
EGF 6 EGF 7
Gm Delta 1381CGCAATCCTTGCTTGAACGGCGGTACTTGCGCAGACATGGTCAACTCCTTCAAGTGCATC
R N P C L N G G T C A D M V N S F K C I
EGF 7
Gm Delta 1441TGCATGCCGGGTTTCATGGGCGACCTGTGCCAGACCAACATGGACGAATGCCTGTTCCAG
C M P G F M G D L) (C Q T N M D E C L F Q
EGF 7 EGF 8
Gm Delta 1501CCCTGCGCCAACGGAGCCACCTGCCACGACCTGGTGCACGACTTCAGGTGCACCTGCAAG
P C A N G A T C H D L V H D F R C T C K
EGF 8
Gm Delta 1561CCCGGCTTCACCGGCAAGGACTGCTCGGTGAACGTGAACGAGTGCGCCAGCTCACCGTGC
P G F T G K D C) (S V N V N E C A S S P C
EGF 8 EGF 9
Gm Delta 1621TTCAACGGTGCCACTTGCCTGGACCGGGTCAACGGGTTCCGGTGCTCTTGCTCGCAGGGT
F N G) (A T C L D R V N G F R C S C S Q G
putative transmembrane domain
Gm Delta 1681TACACGGGGCTGTTGTGCGAGAGTCCTCTAACGGAGGCGGTCAACAACACCTCCTCGAAG
Y T) G L L C E S P L T E A V N N T S S K
Gm Delta 1741GCCGCCGCCTCGGACAGTGGTAGCGGTAGCAGTAGGAACTTTACGGGCAGCGCTTCGTCT
A A A S D S G S G S S R N F T G S A S S
Gm Delta 1801TCTTCGTACGTGGCCGAGTCGCTGAGTACCCAGCAGGTGATCGTGGTGGCCACTCTGGTG
S S Y V A E S L S T Q Q V I V V A T L V

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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Delta 1861CCCGTTGTGATTGTGGTGTTGGTCGGTGTGGCGTGTTACCTGGGACTGAGACACCGGCGT
                  P  V  V  I  V  V  L  V  G  V  A  C  Y  L  G  L  R  H  R  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Delta 1921GCCAAGAAGGAGCAGCGGANGCACGACGAGAAGGTTCGCCACCAGAACGAGCACAATGCC
                 A  K  K  E  Q  R  X  H  D  E  K  V  R  H  Q  N  E  H  N  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Delta 1981GTGCACGGCATGAACAACAAGTGCCTCGACAATCAAATCGTGAACGTGCTAGTGGACAGT
                 V  H  G  M  N  N  K  C  L  D  N  Q  I  V  N  V  L  V  D  S
                ....|....|....|.
Gm Delta 2041AAGCACAAGTGCAACA 2056
                 K  H  K  C  N
```

***Intermediate neuroblasts defective* homolog**
Glomeris marginata

The homeobox is underlined and bold.

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                      10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  1     ATCGACTATTCAGCGTCCGGACCCACTTCACTGTGTTTGCCATACTTGCCCGTTCGGAGA
                I  D  Y  S  A  S  G  P  T  S  L  C  L  P  Y  L  P  V  R  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  61    CTTATGGGACACCCTGTTCTCGCAGGCGCTAATACGAAGACGAAATCGGTGGAAAGTGGA
                L  M  G  H  P  V  L  A  G  A  N  T  K  T  K  S  V  E  S  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  121   ATTGGAACGGGAACGAGATCAGGAACGGAAACGGAGCGAGGTGTTGACGAGGAAACGGAA
                I  G  T  G  T  R  S  G  T  E  T  E  R  G  V  D  E  E  T  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  181   GTGGATCAAGATTCGGACACTATGCTGGACGTTGTGGGAGTGGGTGGGGCTGGAGATGAG
                V  D  Q  D  S  D  T  M  L  D  V  V  G  V  G  G  A  G  D  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  241   AAAGGTGGAGGAGACGGAGGAGGAGGcggaggAGGAGGAAAAGGGAGCAACAAGCGCATG
                K  G  G  G  D  G  G  G  G  G  G  G  G  K  G  S  N  K  R  M
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  301   CGCACTGCGTTCACGAGCACCCAGCTGCTGGAACTGGAACGGGAATTCGCGAGCAACATG
                R  T  A  F  T  S  T  Q  L  L  E  L  E  R  E  F  A  S  N  M
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  361   TACCTGTCGCGCCTGCGCAGAATCGAGATTGTCACTTACCTGAAGTTGAGCGAGAAACAG
                Y  L  S  R  L  R  R  I  E  I  V  T  Y  L  K  L  S  E  K  Q
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  421   GTGAAGATTTGGTTCCAGAACCGAAGGGTCAAGTTCAAGAAGGAAGGTTCTTGCAGTGCT
                V  K  I  W  F  Q  N  R  R  V  K  F  K  K  E  G  S  C  S  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  481   GTAGATGACCGCAAGGATGGGTGCACCAAGTGTCTGTTAAGAGCGTGCTCCAAACGCCCC
                V  D  D  R  K  D  G  C  T  K  C  L  L  R  A  C  S  K  R  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  541   AAAACGCCTCCGCAACGCACCAAAGGCGGAGAATCGCCTTAAAAAACCCAAATAAACAAT
                K  T  P  P  Q  R  T  K  G  G  E  S  P  *
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  601   CAAAAGTGCCCTAGAcGGCGCTCCAGTGAACGGACACAAACGGTTGTGCCTTGTCTTATA
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm ind  661   TAATTATACAGGAACAAGCGTGTCTTTTTTATATACATATACACGAGTGTGTGTGTGTGT
                ....|....|....|....|....|....|....|....|....
Gm ind  721   GCAGTGTGAATACAATTGGTGAATATGCGGATTAAACGAGTGATCCATG 769
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***Islet* homolog**
Glomeris marginata

Conserved domains are bold and underlined. The two LIM domains are followed by the homeodomain and the ISD (islet-specific-domain).

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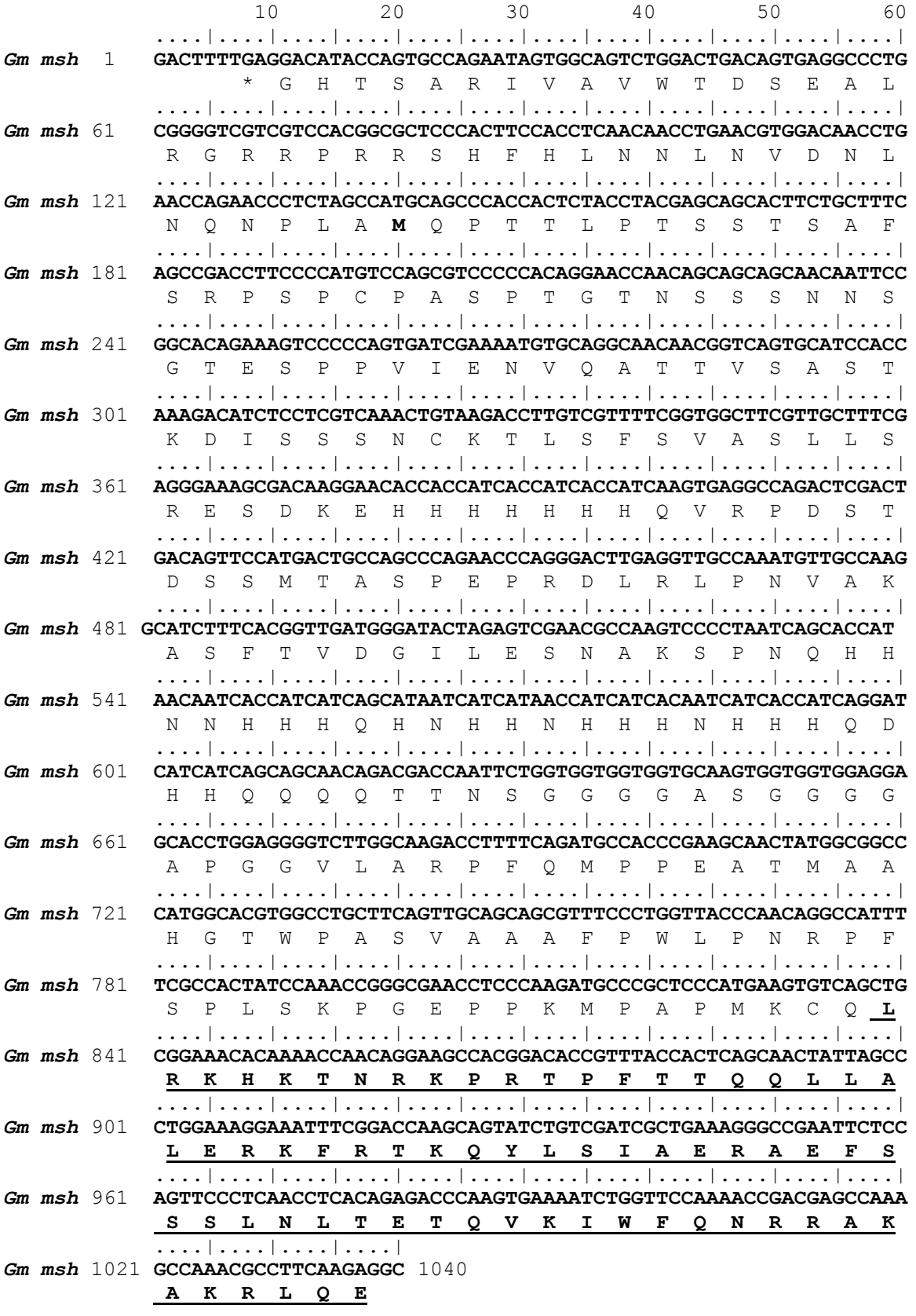
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm islet    1   AGGGATGGGAAGACGTACTGCAAGAGAGACTACGTTAGATTATTTGGAGCGAAGTGCGCC
                 R  D  G  K  T  Y  C  K  R  D  Y  V  R  L  F  G  A  K  C  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is      61   AAGTGCAATTTGGGATTCAGCAAGAACGACTTCGTGATGAGGGCACGGAATAAGATCTAC
                 K  C  N  L  G  F  S  K  N  D  F  V  M  R  A  R  N  K  I  Y
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     121   CGCATAGACTGTTTCCGGTGCATGGCTTGCAGTCGTCAGTTAATACCTGGTGATGAATTT
                 R  I  D  C  F  R  C  M  A  C  S  R  Q  L  I  P  G  D  E  F
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     181   GCACTGAGGGACGATGGCCTGTTCTGCAAGGCAGATCATGAGGTGCTGGAGAAGACGTCT
                 A  L  R  D  D  G  L  F  C  K  A  D  H  E  V  L  E  K  T  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     241   CCAACGAGTGGAGGTGGCAGTGGAGGAAGTGGAAGCGGTGGTGGGAGTGGAGGTGCAGGT
                 P  T  S  G  G  G  S  G  G  S  G  S  G  G  G  S  G  G  A  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     301   GGAGCAGGTGGTGTTGCAGGCGTTGGTGGAAGTGCCAGTGATGTTGATGGTGCTACCGTC
                 G  A  G  G  V  A  G  V  G  G  S  A  S  D  V  D  G  A  T  V
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     361   AATGGTACCAAAGGAACTGGTGTTGGGGCCAATGGCAATGGACAAAATGGACACCATCCT
                 N  G  T  K  G  T  G  V  G  A  N  G  N  G  Q  N  G  H  H  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     421   GGACAGAATGGGCTCCAACTTGCAGCGAACGCAGAGCCAATGGCTACCAACCGGCAAAGG
                 G  Q  N  G  L  Q  L  A  A  N  A  E  P  M  A  T  N  R  Q  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     481   GACTCTGTTCGACCTCAAGTGCATAAGAATCCGGAACAGAAGACGACAAGGGTTCGAACA
                 D  S  V  R  P  Q  V  H  K  N  P  E  Q  K  T  T  R  V  R  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     541   GTGCTCAACGAGAAACAACTTCACACACTTCGGACATGTTACGCGGCAAACCCAAGGCCC
                 V  L  N  E  K  Q  L  H  T  L  R  T  C  Y  A  A  N  P  R  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     601   GACGCGCTCATGAAGGAACAATTGGTGGAGATGACCGGTCTGAGTCCCAGGGTGATCCGG
                 D  A  L  M  K  E  Q  L  V  E  M  T  G  L  S  P  R  V  I  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     661   GTGTGGTTTCAGAACAAGAGGTGTAAGGATAAAAAGAAGAGCATCCTCATGAAGCAGATT
                 V  W  F  Q  N  K  R  C  K  D  K  K  K  S  I  L  M  K  Q  I
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     721   CAGCAGCAGGAGAAGGATGGAAGAAGGTTACCGGGACTTGCGGGTATGCATGGTGTTCCA
                 Q  Q  Q  E  K  D  G  R  R  L  P  G  L  A  G  M  H  G  V  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm is     781   ATGATAGCTTCGAGTCCTGTTCGCCACGAGTCGCCCCTGCAAGTGAACCCCGTAGAAGTG
                 M  I  A  S  S  P  V  R  H  E  S  P  L  Q  V  N  P  V  E  V
                ....|....|....|....|....|....
Gm is     841   CAAAGCTACCAGCCCCCTTGGAAAGCCCT 869
                 Q  S  Y  Q  P  P  W  K  A
```

***Msh* homolog**
Glomeris marginata

The homeobox region is bold and underlined.



***Notch* homolog, Accession number AJ36344**
Archispirostreptus species

The EGF repeats are underlined and in italics.

```
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 1      AACTTCGACGACTGCGCCGGCGCGGCCTGCTTCAACGGTGCCACTTGCCACGACCGAGTG
                 N  F  D  D  C  A  G  A  A  C  F  N  G  A  T  C  H  D  R  V
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 61     GGCTCCTTCTACTGCCAATGTCCACCCGGCAAAACGGGCCTGCTCTGCCACCTGGACGAC
                 G  S  F  Y  C  Q  C  P  P  G  K  T  G  L  L  C  H  L  D  D
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 121    GCCTGCGCCAGCAATCCATGCCACGCCAGTGCCCTTTGCGACACCAATCCCATTGACGGG
                 A  C  A  S  N  P  C  H  A  S  A  L  C  D  T  N  P  I  D  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 181    ACGTACATCTGTTCCTGTCCGAGTGGCTATAAGGGCATTGACTGCACCAAGAACATCGAT
                 T  Y  I  C  S  C  P  S  G  Y  K  G  I  D  C  T  K  N  I  D
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 241    GAGTGTGAAGAAGGTTCTCCTTGTGAACACGATGGCAAATGCGTCAACACTCCTGGCTCA
                 E  C  E  E  G  S  P  C  E  H  D  G  K  C  V  N  T  P  G  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
As Notch 301    TTCAGCTGCAACTGCACCAAAGGGTTCACCGGTCCACGGTGCGAGGTCAACATCAACGAG
                 F  S  C  N  C  T  K  G  F  T  G  P  R  C  E  V  N  I  N  E
                ....|...
As Notch 361    TGCGACTA 368
                 C  D
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***Notch* homolog, Accession number AJ36342**
Glomeris marginata

The EGF domains are underlined and in italics.

```
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch     1  GAGGAAATGTCGCTCGGATTTAAATTAACACGCATCGCTATCTTCATCATCATCATCATC
                 E  E  M  S  L  G  F  K  L  T  R  I  A  I  F  I  I  I  I  I
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch    61  ATTGGCTTCTCTGCGACAGTCGACGGACTTGTTTCCTGCTCCCCTAACCCCTGCAACAAT
                 I  G  F  S  A  T  V  D  G  L  V  S  (C  S  P  N  P  C  N  N
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   121  GGCGCCACCTGTGTGGCGGTCAAAGGCGAGGCCTATGCTTGCAGCTGCCCTTCAATGTAC
                 G  A  T  C  V  A  V  K  G  E  A  Y  A  C  S  C  P  S  M  Y
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   181  CTGGGCGAGTATTGTCAACACGTGAACCCATGCCATACGGGTCCTGGTCCTCGATGCCAG
                 L  G  E  Y  C  Q  H)  V  N  P  (C  H  T  G  P  G  P  R  C  Q
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   241  AACGGAGGAACGTGTCACGTGTTACTCAACGCCACTTCCGGTCTACGATTCTCCCGCACG
                 N  G  G  T  C  H  V  L  L  N  A  T  S  G  L  R  F  S  R  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   301  TGTCCTGTGGGTTTTTCGGCGTCATTGTGCGAGATCCCAGTGCCGAACTCCTGCGACAGC
                 C  P  V  G  F  S  A  S  L  C  E  I)  P  V  P  N  S  (C  D  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   361  GACCCTTGCCTCAATGGAGGCACCTGCACCCTCAGGACCCTCACCAACTACACGTGCGCA
                 D  P  C  L  N  G  G  T  C  T  L  R  T  L  T  N  Y  T  C  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   421  TGCGCCACAGGATTCAGAGGCAAACACTGCGAATATGTGGACCACTGCGCACCTCAGCCA
                 C  A  T  G  F  R  G  K  H  C  E)  Y  V  D  (H  C  A  P  Q  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   481  TGCAGAAACGGAGCAACTTACCGGTCAGTAGATGACACGTTCAGATGCAAATGCCGAGAA
                 C  R  N  G  A  T  Y  R  S  V  D  D  T  F  R  C  K  C  R  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   541  GGATTCGCAGGCAGCGCGTGCACCGATGACATCGACGAGTGCAGCAGACACAGTCCTTGC
                 G  F  A  G  S  A  C  T  D)  D  I  D  E  (C  S  R  H  S  P  C
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   601  AAGCAGGGTTCTTGCATCAACACCATCGGCTCTTACAGACGCACTTGTGAACAGGGATTC
                 K  Q  G  S  C  I  N  T  I  G  S  Y  R  R  T  C  E  Q  G  F
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   661  ACCGGCCAGAACTGCGAAAGTAAATATATTCCTTGCGAACCAAGTCCGTGCCACAATGGT
                 T  G  Q  N  C  E  S)  K  Y  I  P  (C  E  P  S  P  C  H  N  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   721  GGCTCGTGTCAACCTGTTGACACACTCAACTACAAGTGTACCTGTCCATCTGGACTCACT
                 G  S  C  Q  P  V  D  T  L  N  Y  K  C  T  C  P  S  G  L  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   781  GGCAACAACTGCGAGGTGAACATTGATGACTGTCCCGGTAATCTTTGCCAAAATGGTGCC
                 G  N  N  C  E  V)  N  I  D  D  (C  P  G  N  L  C  Q  N  G  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   841  ACTTGTGTTGATGGAGTCAATTCGTATACCTGCCAATGTCCGCCAACGTATTTAGGAACG
                 T  C  V  D  G  V  N  S  Y  T  C  Q  C  P  P  T  Y  L  G  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   901  TACTGCACGAAGGACGTTGATGAGTGTGGTCTACGCCCAAGTGTTTGCAAGAATGGAGCT
                 Y  C  T)  K  D  V  D  E  C  G  L  R  P  S  V  C  K  N  G  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch   961  ACGTGTACCAATAGCATCGGTGGTTACAACTGCATATGCGTAAATGGGTGGACTGGCCGC
                 T  C  T  N  S  I  G  G  Y  N  C  I  C  V  N  G  W  T  G  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch  1021 GACTGCAGCGAGAACATCGATGGCTGCGCGGTCGCGGCTTGCTTCAACGGCGCAACGTGC
                 D  C  S  E)  N  I  D  G  (C  A  V  A  A  C  F  N  G  A  T  C
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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch 1081CAAGATCGAGTTGGGTCCTTCGCTTGTCACTGTCCGCCAGGAAAAACCGGGCTTTTGTGT
                  Q  D  R  V  G  S  F  A  C  H  C  P  P  G  K  T  G  L  L  C
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch 1141CATTTGGACGATGCGTGCACAAGTAATCCGTGCCATGCGAGCGCGATCTGCAACACGAGC
                  H  L) D  D  A (C  T  S  N  P  C  H  A  S  A  I  C  N  T  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch 1201CCGATCGACGGTGGATACATCTGCTCATGTCCCAGTGGATATAAAGGAGTCGACTGCACA
                  P  I  D  G  G  Y  I  C  S  C  P  S  G  Y  K  G  V  D  C  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm Notch 1261GAAGATATCAACGAATGTGAAGAAGGTTCACCATGCGAACACGGAGGGCTTTGCGTCAAC
                  E) D  I  N  E (C  E  E  G  S  P  C  E  H  G  G  L  C  V  N
                ....|....|....|....|....|....|.
Gm Notch 1321ACTCCAGGCTCGTTCCGTTGCAATTGCACCA 1351
                  T  P  G  S  F  R  C  N  C  T
```

***Numb* homolog**
Glomeris marginata

The nucleotides missing from one transcript are in italics. The conserved Phosphotyrosine interaction domain (PID) is underlined and in bold type.

```
                    10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  1     GTGGATCAGACCATAGAGAAGGTTTCATTCTGCGCTCCTGACCGCAACCATGGGAAGGGC
                V  D  Q  T  I  E  K  V  S  F  C  A  P  D  R  N  H  G  K  G
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  61    TTTTCGTACATTTGCCGAGATGGTACTACAAGACGATGGATGTGCCATGGCTTCTTAGCC
                F  S  Y  I  C  R  D  G  T  T  R  R  W  M  C  H  G  F  L  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  121   GTCAAGGATTCGGGTGAGAGGTTAAGTCATGCCGTGGGGTGCGCGTTTGCTGCATGCTTG
                V  K  D  S  G  E  R  L  S  H  A  V  G  C  A  F  A  A  C  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  181   GAGAGAAAGCAAAAACGAGACAAAGAATGTGGTGTTACCATGACATTTGACCCCAATGGG
                E  R  K  Q  K  R  D  K  E  C  G  V  T  M  T  F  D  P  N  G
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  241   TCAGCATTTACGCGGACAGGATCATTTCGCCAAACATCTTTGACGGAGAGGATGCAGGAT
                S  A  F  T  R  T  G  S  F  R  Q  T  S  L  T  E  R  M  Q  D
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  301   CCTCAAGAATGCAAACCTTTTGATCCTCCTCCTCCGAAGAACATAACAAACCCATTTGCC
                P  Q  E  C  K  P  F  D  P  P  P  P  K  N  I  T  N  P  F  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  361   ATTGAGAGGCCTCATGCGACACCTATGATGCTGGAACGGCAAGGCTCGTTTCGTGGATTT
                I  E  R  P  H  A  T  P  M  M  L  E  R  Q  G  S  F  R  G  F
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  421   AACCAGCTTCAGCAGGCATCACCTTTTAAGCGACAGTTGTCACTGCGTGTTAACGATCTG
                N  Q  L  Q  Q  A  S  P  F  K  R  Q  L  S  L  R  V  N  D  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  481   CCCTCGAACCTAGAAAGACAACAGAAGTCGCTGAGTTCACGGCCTTGTGAAAAGCATGAG
                P  S  N  L  E  R  Q  Q  K  S  L  S  S  R  P  C  E  K  H  E
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  541   GAATCACCTTCCAACTTAAATGGCCTATCGTCGCAGCCAGTCCCTGTCCCTGGACCAGCA
                E  S  P  S  N  L  N  G  L  S  S  Q  P  V  P  V  P  G  P  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  601   AGTCCTCAACTCCCTCCTCGTATTCCTAGGAACAGGCTTCATTCATTGCCAAACACAGCA
                S  P  Q  L  P  P  R  I  P  R  N  R  L  H  S  L  P  N  T  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  661   GTCAACATGAGCCCGATCCCTGAAAGTTCTCCAACTGGTGACAGACAGCCTGACACTATT
                V  N  M  S  P  I  P  E  S  S  P  T  G  D  R  Q  P  D  T  I
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  721   CGTGCCATGTGCCAGCAGGTTACTGAAGGACTTTCCATGTTGTGCTCCACAGATGATCCT
                R  A  M  C  Q  Q  V  T  E  G  L  S  M  L  C  S  T  D  D  P
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  781   TTTACGCCATCCCGTGCCACATCACAAAATACTCGTTTAGCTGGACCAAGTGTCGGTATG
                F  T  P  S  R  A  T  S  Q  N  T  R  L  A  G  P  S  V  G  M
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  841   AACAACCATGTGGCTGTGGAGTCGTTTGAACCAATTATAGAGCAAAACACCTGGGAGCCA
                N  N  H  V  A  V  E  S  F  E  P  I  I  E  Q  N  T  W  E  P
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  901   GCGAAAGAAGGTGCAAGTCGAGCTGAAGCATGGCTTGCCAGTAACGTGCCGTCCCACATT
                A  K  E  G  A  S  R  A  E  A  W  L  A  S  N  V  P  S  H  I
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  961   TCTGTTAGCAAATCATCGTCCAACCCGCTTGATTTATTCGGGGAAGCTCGAACCAATGGT
                S  V  S  K  S  S  S  N  P  L  D  L  F  G  E  A  R  T  N  G
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb  1021  CAAACAAGTCCGCACTCGCCATCCATGGTGACTGCTCCTCCCCGCAAAGCGCCACATCTG
                Q  T  S  P  H  S  P  S  M  V  T  A  P  P  R  K  A  P  H  L
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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm numb 1081 TCTCACCTGCGCAGTCAGTCTCTTGGTGCAAGTGCTGATCCGCACGTGTCTCAAGCGCAT
              S   H   L   R   S   Q   S   L   G   A   S   A   D   P   H   V   S   Q   A   H
                ....|....|....|....|....|.
Gm numb 1141 CCTGTGCAGTCACCGCCATCCATCGC 1166
              P   V   Q   S   P   P   S   I
```

***Pannier*, two original fragments**
Glomeris marginata

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                  10        20        30        40        50        60
              ....|....|....|....|....|....|....|....|....|....|....|
Gm pan     1  GGGGCAATATCTGTGCAATGCTTGTGGTCTTTATCACAAAATGAATGGCATGAATAGACC
Gm pan 2   1  T-------------------------------------------C--------C--G--

                  70        80        90       100       110       120
              ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan     61  ATTAATCAAGCCCCAGAAGAGACTGCATGCTAACCGGCGCATTGGACTTTCTTGTTCCAA
Gm pan 2   61  -C--G-G--A--T--A-G-------AC-------C-AA-G-TG--CT-G-GC---A-A--

                 130       140       150       160       170       180
              ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan    121  CTGTGGGACTGCGACGACCTCCTTATGGCGAAGGAATAATCAAGGAGAACCTGTTTGCAA
Gm pan 2  121  T--C-CA--GA-C--C---A-------------------------------------

                 190       200       210       220
              ....|....|....|....|....|....|....|....|
Gm pan    181  CGCATGTGGCCTTTATTTCAAGCTACATGGCCCAAACCGCCCTCT 225
Gm pan 2  181  ----------------------------A--C--------A-- 225
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***Pannier*, RACE fragment.**
Glomeris marginata

The conserved zinc fingers 1 and 2 are underlined and in bold type.

```
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  1       GTGTGTCAGATGTGGGCATCGAAGGTCAGTAGTCTCCGGTCACATGAACAGGCAGAAGCG
                 V  C  Q  M  W  A  S  K  V  S  S  L  R  S  H  E  Q  A  E  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  61      AGTTTGGAAACGGACAGCAGTGACTGTTCCTCCTCCACTTTGTTGACGGACAACACCACG
                 S  L  E  T  D  S  S  D  C  S  S  S  T  L  L  T  D  N  T  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  121     GCCACTGTCCTCGTTTCATCAGGGTACTCGGACCCTTACGGGTTCCCGATGCCCCAACCC
                 A  T  V  L  V  S  S  G  Y  S  D  P  Y  G  F  P  M  P  Q  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  181     AACTTGACCCACGGCCACGTGCCACTACCATCTGCATCCTCTGCATCGGGACAATCTTCT
                 N  L  T  H  G  H  V  P  L  P  S  A  S  S  A  S  G  Q  S  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  241     GCACCTCCTCACCACGAGGGGCAGCTAAGTTACGCGGGTGTTTTTGCGAGTTTCGGTGGT
                 A  P  P  H  H  E  G  Q  L  S  Y  A  G  V  F  A  S  F  G  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  301     TCCGATGGACCTGGACACCACGATGGCCACATGACCAACAGTTCGGTGTACCTGCCGCAT
                 S  D  G  P  G  H  H  D  G  H  M  T  N  S  S  V  Y  L  P  H
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  361     GCCCCGACGAGGGTCTTCCCCATGGCCTACTTGGGGGCATCTTCCGCCAATGGCAACGGA
                 A  P  T  R  V  F  P  M  A  Y  L  G  A  S  S  A  N  G  N  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  421     AGTCATGGGATCCCGGGAAACAACGAAGGTGCTGCTGGAACGAACAATTCCTCTTGTAAC
                 S  H  G  I  P  G  N  N  E  G  A  A  G  T  N  N  S  S  C  N
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  481     AATGCATCAGCGAGTGGAGGAATGTGGTCACCCATGGTGGGACCTCAAGTGAGCAATGGA
                 N  A  S  A  S  G  G  M  W  S  P  M  V  G  P  Q  V  S  N  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  541     CGGGCGAACGGTGTTGGCCTGATGTACCCGGCACCTTACGCGGAACTCGCGGCGTGGGCC
                 R  A  N  G  V  G  L  M  Y  P  A  P  Y  A  E  L  A  A  W  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  601     ACTTACGATGGCTCTTCTCCGGCTTTTCCGTACTCCATGCAAACTTCAGGACAAGGACCG
                 T  Y  D  G  S  S  P  A  F  P  Y  S  M  Q  T  S  G  Q  G  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  661     CAATGCAGATCTGTTCCAGTGGACGAAGCCTTCTTCGGCGAAGGACGAGAGTGTGTCAAT
                 Q  C  R  S  V  P  V  D  E  A  F  F  G  E  G  R  E  C  V  N
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  721     TGTGGTGCCATTTCCACACCCTTGTGGAGGAGAGGCGGAACTGGACATTATCTGTGCAAT
                 C  G  A  I  S  T  P  L  W  R  R  G  G  T  G  H  Y  L  C  N
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  781     GCTTGTGGTCTTTATCACAAAATGAATGGCATGAATAGACCATTAATCAAGCCCCAGAAG
                 A  C  G  L  Y  H  K  M  N  G  M  N  R  P  L  I  K  P  Q  K
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  841     AGACTGCATGCTAACCGGCGCATTGGACTTTCTTGTTCCAACTGTGGGACTGCGACGACC
                 R  L  H  A  N  R  R  I  G  L  S  C  S  N  C  G  T  A  T  T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  901     TCCTTATGGCGAAGGAATAATCAAGGAGAACCTGTTTGCAACGCATGTGGCCTTTATTTC
                 S  L  W  R  R  N  N  Q  G  E  P  V  C  N  A  C  G  L  Y  F
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  961     AAGCTACATGGGGTAAGTCGGCCATTAGCCATGAAGAAAGACAGCATTCAAACTAGGAAA
                 K  L  H  G  V  S  R  P  L  A  M  K  K  D  S  I  Q  T  R  K
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  1021    CGAAAACCAAAGAGTACACAGTCTCCTGTAAAAGTTGAAGTTTCTTCTGAAAGTCACCCA
                 R  K  P  K  S  T  Q  S  P  V  K  V  E  V  S  S  E  S  H  P
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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  1081 GCAACTCCTAAAGTGATGGAAAATGCTTCTTCAGGACATATTGACATGAAGCCAGTTACC
              A   T   P   K   V   M   E   N   A   S   S   G   H   I   D   M   K   P   V   T
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pan  1141 GCATCTTCCAGTCTGTACAGCATGCCACTATCTTCATCTTCAGCTTCGAACTTGTTTTAC
              A   S   S   S   L   Y   S   M   P   L   S   S   S   S   A   S   N   L   F   Y
                ....|....|....|....|....|....|....|....|....
Gm pan  1201 CCGCAATCAAAATCGGTGACGTCACCATCACCAGCGTTGTCCAC 1244
              P   Q   S   K   S   V   T   S   P   S   P   A   L   S
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***Prospero* homolog**
Glomeris marginata

The conserved domains are underlined. The homeodomain is in bold type followed by the propero domain which is in italics.

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                    10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  1     AGTCTGCATTCGGCACTGATGGCCGCCACAGGGTTACCGTCAGACACCCTTCATATGTTG
                S  L  H  S  A  L  M  A  A  T  G  L  P  S  D  T  L  H  M  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  61    CACGCAATCAAGGGAGAAAATGGAGATGTGTCCGACTGCAATTCCGGAGACAACCAGTAC
                H  A  I  K  G  E  N  G  D  V  S  D  C  N  S  G  D  N  Q  Y
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  121   GATGGTCCAACACCTACTTCTTCCACGTTGACTCCAATGCATCTGCGCAAGGCCAAGCTG
                D  G  P  T  P  T  S  S  T  L  T  P  M  H  L  R  K  A  K  L
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  181   ATGTTCTTCTACGTAAGATACCCAAGTTCGGCCATCTTGAAAATGTACTTCCCCGACATC
                M  F  F  Y  V  R  Y  P  S  S  A  I  L  K  M  Y  F  P  D  I
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  241   AAGTTCAACAAAAACAACACGGCTCAGCTCGTCAAATGGTTCTCCAACTTCCGGGAATTT
                K  F  N  K  N  N  T  A  Q  L  V  K  W  F  S  N  F  R  E  F
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  301   TATTATATCCAAATGGAGAAATATGCTCGTCAAGCGATCAGTGAAGGTGTGAAATCGTGT
                Y  Y  I  Q  M  E  K  Y  A  R  Q  A  I  S  E  G  V  K  S  C
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  361   GAAGACATCGCAGTTAGTTTTGATTCGGAGCTGTACAGAGTCCTCAACCTTCATTACAAC
                E  D  I  A  V  S  F  D  S  E  L  Y  R  V  L  N  L  H  Y  N
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  421   CGCAACAACCACATCGAGGTGCCGACCAACTTCCGGTACGTTGTGGAGCAGACGTTGAGG
                R  N  N  H  I  E  V  P  T  N  F  R  Y  V  V  E  Q  T  L  R
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  481   GAGTTCTTCAAGGCCATAATGGCGGGCAAGGACACGGAGCAGTCGTGGAAGAAGGCCATC
                E  F  F  K  A  I  M  A  G  K  D  T  E  Q  S  W  K  K  A  I
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  541   TACAAAGTGATAGCTCGGCTCGATGATTCGTTGCCCGAGTACTTCAAGTCTCCAAACTTC
                Y  K  V  I  A  R  L  D  D  S  L  P  E  Y  F  K  S  P  N  F
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm pros  601   CTGGAACAACTTGAATGACGTCGGGCCTTGATCGTCCAGAACCAACAGCACCAATCAACA
                L  E  Q  L  E  *
               ....|....|....|....|....|....|....|....|....|....|....|...
Gm pros  661   AGCGCCAGCTCCTGTGATAAACTAGCACTTGCTTCTTTCTTTCTCCTTTTCTCCGCTTG
719
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***Snail* homolog**
Glomeris marginata

The conserved zinc finger regions are underlined and in bold type.

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                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  1    AAGTCACCATCACCTCTCCATCATTCGCTGCACAACACAGATGATGGCTCATCTATCGGA
             K  S  P  S  P  L  H  H  S  L  H  N  T  D  D  G  S  S  I  G
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  61   CCAGAGGATTTGTCAGTGCGTTCGGCAACACCATCGCCATCACCTCCGATGGTGCTGGAA
             P  E  D  L  S  V  R  S  A  T  P  S  P  S  P  P  M  V  L  E
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  121  CCACTAAGCCTAAACGTGCATCACCATCCACAATCTCCCGGCAACCCACGGCCTTCTCTG
             P  L  S  L  N  V  H  H  H  P  Q  S  P  G  N  P  R  P  S  L
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  181  CACCACCCAACCGCAATCTACCACCAAAACAGCCACAGTCAATACCTCCAGAGCTACCTA
             H  H  P  T  A  I  Y  H  Q  N  S  H  S  Q  Y  L  Q  S  Y  L
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  241  TCCTCCTACATCTACCACGACCATCAACACCACCATCACCATTTTCACAGCCGATCACCA
             S  S  Y  I  Y  H  D  H  Q  H  H  H  H  H  F  H  S  R  S  P
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  301  GTGTACCAACCAACCCCGGTCATTGCCCGACACCCTTTGATGACCACGTCCATCCCGCAG
             V  Y  Q  P  T  P  V  I  A  R  H  P  L  M  T  T  S  I  P  Q
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  361  CAGCAACAGCATCACCAGACGATTGTTGCAGATGCGGATGAAGACGAGGACATGACCATC
             Q  Q  Q  H  H  Q  T  I  V  A  D  A  D  E  D  E  D  M  T  I
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  421  GTCGAGGAAGCACCTCCTAGCCCCTACAGCAGCAGTGCCAGTACAACCAGCAAGGACCCA
             V  E  E  A  P  P  S  P  Y  S  S  S  A  S  T  T  S  K  D  P
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  481  TGTGACATTGATAAACGCAAGGAATTGGATGATACCACCGTTGACATTACTCGAGGTGCC
             C  D  I  D  K  R  K  E  L  D  D  T  T  V  D  I  T  R  G  A
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  541  TCTAAAGGATCTCAAGGTCAAGGCCGGTNTAAAAGGTACCAGTGCCCAGATTGTGGAAAG
             S  K  G  S  Q  G  Q  G  R  X  K  R  Y  Q  C  P  D  C  G  K
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  601  AGTTACTNTACTGTGAGTGGGGTTTCGAAGCATCAGCAGTTCCACTGCGGTCCTGCGGCA
             S  Y  X  T  V  S  G  V  S  K  H  Q  Q  F  H  C  G  P  A  A
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  661  AGGAGGTCGTTTTCCTGCAAGTTCTGCGAAAAGGTGTACGTGTCTTTGGGGGCACTGAAG
             R  R  S  F  S  C  K  F  C  E  K  V  Y  V  S  L  G  A  L  K
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  721  ATGCACATTCGCACCCATACGCTTCCCTGCAAGTGTGCCTTGTGTGGTAAAGCCTTCTCC
             M  H  I  R  T  H  T  L  P  C  K  C  A  L  C  G  K  A  F  S
            ....|....|....|....|....|....|....|....|....|....|....|....|
Gm sn  781  AGGCCCTGGTTACTGCAAGGACATATTCGGACTCACACTGGCGAGAAGCCCTTCTCCTGT
             R  P  W  L  L  Q  G  H  I  R  T  H  T  G  E  K  P  F  S  C
            ....|....|....|....|..
Gm sn  841  CAACATTGCCAGAGAGCCTTCG 862
             Q  H  C  Q  R  A  F
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Sox homologs
Glomeris marginata

HMG domains of six original fragments obtained.

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                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1    1   TCACGTGGTCAGCGGAGGAAAATGGCGCAGGAGAATCCGAAGATGCACAACTCCGAGATA
                 S  R  G  Q  R  R  K  M  A  Q  E  N  P  K  M  H  N  S  E  I
Gm SoxB2    1   TCACGTGCCCAAAGACGCAAGATCGCGCAGGAAAACCCGAAGATGCACAACTCTGAGATC
                 S  R  A  Q  R  R  K  I  A  Q  E  N  P  K  M  H  N  S  E  I
Gm SoxB3    1   TCACGTGCCCAAAGACGCAAGATCGCGCAGGAAAATCCGAAGATGCACAACTCCGAGATA
                 S  R  A  Q  R  R  K  I  A  Q  E  N  P  K  M  H  N  S  E  I
Gm SoxC1    1   AGCCAGATTGAACGGAGGAAGATCTGCGAACAGCAACCGGACATGCACAACGCCNAGATA
                 S  Q  I  E  R  R  K  I  C  E  Q  Q  P  D  M  H  N  A  X  I
Gm SoxE1    1   GCTAAAGTGGAAAGAAAGAGGCTTGCTGATGAAAATCCAGATCTTCATAATGCGGACCTG
                 A  K  V  E  R  K  R  L  A  D  E  N  P  D  L  H  N  A  D  L
Gm SoxE2    1   GCACAAGCTGCAAGACGCAAGTTGGCCGATCACTACCCACATCTGCACAATGCTGAACTC
                 A  Q  A  A  R  R  K  L  A  D  H  Y  P  H  L  H  N  A  E  L

                    70        80        90       100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1   61   AGTAAGCGACTGGGAGCCGAGTGGAAGTTGCTGTCAGAAGCGGAAAAGAGGCCGTTTATT
                 S  K  R  L  G  A  E  W  K  L  L  S  E  A  E  K  R  P  F  I
Gm SoxB2   61   AGCAAGAGGTTAGGGGCCGAGTGGAAACTGCTGACCGAATCCGAGAAGAGACCTTTCATA
                 S  K  R  L  G  A  E  W  K  L  L  T  E  S  E  K  R  P  F  I
Gm SoxB3   61   AGTAAGCGACTGGGAGCCGAGTGGAAGTTGCTGTCAGAAGCGGAAAAGAGGCCGTTTATT
                 S  K  R  L  G  A  E  W  K  L  L  S  E  A  E  K  R  P  F  I
Gm SoxC1   61   AGCAAGCGGCTGGGAAAACGCTGGAAAATGCTGAGCGACGTCNAGCGACAACCGTTTATC
                 S  K  R  L  G  K  R  W  K  M  L  S  D  V  X  R  Q  P  F  I
Gm SoxE1   61   AGCAAGATGTTGGGTAAAAAATGGCGCAGTCTGACCCCATCCGACCGAAGACCTTACGTA
                 S  K  M  L  G  K  K  W  R  S  L  T  P  S  D  R  R  P  Y  V
Gm SoxE2   61   AGCAAAACTTTGGGCAAACTTTGGAGGTTATTAAGCGACGACGAGAAACGGCCGTTCATC
                 S  K  T  L  G  K  L  W  R  L  L  S  D  D  E  K  R  P  F  I

                   130       140
                ....|....|....|....|....|..
Gm SoxB1  121   GACGAGGCAAAGCGACTAAGGGCCGTT 147
                 D  E  A  K  R  L  R  A  V
Gm SoxB1  121   GACGAAGCGAAACGTCTGAGGGCCCAA 147
                 D  E  A  K  R  L  R  A  Q
Gm SoxB3  121   GACGAGGCAAAGCGACTAAGGGCCGTT 147
                 D  E  A  K  R  L  R  A  V
Gm SoxC1  121   GAANAAGCTGAGCGACTGCGCATCCTT 147
                 E  X  A  E  R  L  R  I  L
Gm SoxE1  121   GAGGAAGCGGAACGTCTTCGGGTTCTG 147
                 E  E  A  E  R  L  R  V  L
Gm SoxE2  121   GAAGAAGCTGAGCGGCTTCGAGTCATC 147
                  E  E  A  E  R  L  R  V  I
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***Sox B1* homolog, RACE fragment.**
Glomeris marginata

The HMG domain is underlined and in bold font.

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                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1    1   ATGAATGCATTCATGGTGTGGTCACGTGGTCAGCGGAGGAAAATGGCGCAGGAGAATCCG
                 M  N  A  F  M  V  W  S  R  G  Q  R  R  K  M  A  Q  E  N  P
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1   61   AAGATGCACAACTCCGAGATAAGTAAGCGACTGGGAGCCGAGTGGAAGTTGCTGTCGGAA
                 K  M  H  N  S  E  I  S  K  R  L  G  A  E  W  K  L  L  S  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  121   GCGGAAAAGAGGCCGTTTATTGACGAGGCAAAGCGACTAAGGGCCGTTCACATGAAAGAA
                 A  E  K  R  P  F  I  D  E  A  K  R  L  R  A  V  H  M  K  E
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  181   CACCCTGACTACAAGTACCGGCCGAGACGTAAGACTAAGACGTTGATGAAGAAAGACAAG
                 H  P  D  Y  K  Y  R  P  R  R  K  T  K  T  L  M  K  K  D  K
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  241   TACCCACTGGCAGGTGGACTGTTACCGGCAGACCCCGTGAGAGCACCAGTGCAGCAAGTG
                 Y  P  L  A  G  G  L  L  P  A  D  P  V  R  A  P  V  Q  Q  V
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  301   GGCAGGGAGATGTACCAGATGAACGGCTACATGCCTAATGGCTACCCCAGCATGATGCAC
                 G  R  E  M  Y  Q  M  N  G  Y  M  P  N  G  Y  P  S  M  M  H
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  361   GCAGCTCACGACGTACCACATGCCTACCAGCAACATGCAGCAGCGGCGGCGTCCTTCAGT
                 A  A  H  D  V  P  H  A  Y  Q  Q  H  A  A  A  A  A  S  F  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  421   GCTGCTGCAGCAGCAGCAGCCAACAACCAGATGGCCACCAACGGTAGCCTTTACCCACGG
                 A  A  A  A  A  A  A  N  N  Q  M  A  T  N  G  S  L  Y  P  R
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  481   TACGACATGACTGCCAGCATGCACTCGTCACCGATGACCACATGCTCATCCGGTTCCATG
                 Y  D  M  T  A  S  M  H  S  S  P  M  T  T  C  S  S  G  S  M
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  541   TCGTCTTACATGAACGGCAGTGCTAACTACTCGATGGCAATGTCATCACCTTATTCGTCG
                 S  S  Y  M  N  G  S  A  N  Y  S  M  A  M  S  S  P  Y  S  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  601   ATGCAGTCTGCGGCTGCAGCAGCAGCGCAGATGTCGCCAACACTCAAAGGTAGTGACTCT
                 M  Q  S  A  A  A  A  A  A  Q  M  S  P  T  L  K  G  S  D  S
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  661   GGAGGCTCTTCTTCATCGTCTTCTGCAGCCGCAGCCGCCGCAGCCTCCGCTATAGCAGCA
                 G  G  S  S  S  S  S  S  A  A  A  A  A  A  A  S  A  I  A  A
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  721   TCCAAGCGTTCGGAGTACCCTGGAGACCTGAGGCAGATGATAAGCATGTACCTTCCAGGA
                 S  K  R  S  E  Y  P  G  D  L  R  Q  M  I  S  M  Y  L  P  G
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  781   GATGGTAGTGACCCAACGTCTCACCAGGGCAGACTTCTGCAGGCGCATTATCAGAGCCAG
                 D  G  S  D  P  T  S  H  Q  G  R  L  L  Q  A  H  Y  Q  S  Q
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  841   TCGGACGCAATGAGCAGTGCAGTGCCTCCGTTGACGCACATGTGACAGATGCAGCAACAG
                 S  D  A  M  S  S  A  V  P  P  L  T  H  M  *
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  901   CGTTAGACTCAAAGGTGACTCTTATGTACAGGGGGTTGTGTGAGTGTGGCAGAACTGCTG
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1  961   AGAAACGCGTGCAAATGTTGAAGAGCAAAGGGCCAGAGATTGGACCCCAAAGTCTCACCT
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1102   TTCTAACTCCACTCCCTTGGCTTCGTGAGAGGAAAGAAAGTGGCCAACAACTGTAACATA
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1108   ACCTGATGTTGGTTACAGATGTGGCCATCAGAAAGGCCGAGACATGGTCTTCTTCGCTCA
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1114   CATGAAATGCAGATGATCCGAGGTGGGCAACAACTGTGGCATGTGATGACCAACAATACG
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                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1120   TGTCACATGTGGCCGGTAACTGTCGCCGCACTCGTTACAAGATGCTGCGTGTGATTGTAC
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1126   ACTGAGAAGGTGTTGATGGCAGCAAGATAAAGTGTAATGAGGGGAAGAAGTCGATGAAGG
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1132   CAACACTGAAAACGGATCCATCCGACTGAAAAGGGAAGTTGATTGTATAAGATGGCTTCC
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1138   GAGTTTCGAAATTTGTTCGGTTTTTTGCATTATTATAGTTGGCGGTTGCGGCGAAACAGT
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1144   TGCATTTGCATGCGATTCCAGTGTGTCCAATAGCTATATATGATTCTTGTTGGCTTTTTC
                ....|....|....|....|....|....|....|....|....|....|....|....|
Gm SoxB1 1150   ATGATGAGGATTCAAATTGGGGTAAAAAGTATTAAAACGAATTCAGCAAATTGGTTTGGT
                ....|.
Gm SoxB1 1561   TTGAAT 1566
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Tinmann*, amplified instead of *vnd
Glomeris marginata

The highly conserved homeodomain is underlined and in bold type.

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                    10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   1     TCAGCCATCATTGGTGTTCGCAGTGCCCAAAGGCCACAGACCACCATTTCTAAATTCCAA
                S  A  I  I  G  V  R  S  A  Q  R  P  Q  T  T  I  S  K  F  Q
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   61    GTCTGCCTCGATACCGAAAAATCCGAAAATCTGGCGTGGCCATTTGCTCACGAGGGGGCC
                V  C  L  D  T  E  K  S  E  N  L  A  W  P  F  A  H  E  G  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   121   ATGTTGCCAGGAGCCCATTCGGCTGCTATGGCCTCAATCACAACGCCCTTCTCAGTCAAA
                M  L  P  G  A  H  S  A  A  M  A  S  I  T  T  P  F  S  V  K
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   181   GACATCCTCAACTTGGGTGACCCATCTGCAATGGATCCTCAAGCTTTCTTCTTCCAGAGC
                D  I  L  N  L  G  D  P  S  A  M  D  P  Q  A  F  F  F  Q  S
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   241   GCTAGTAGTCTTGAGAAGATGCTCTTCGACGATTCTTGCTCCATAATGATGAATGGTACT
                A  S  S  L  E  K  M  L  F  D  D  S  C  S  I  M  M  N  G  T
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   301   TCGGTTGGACCTGAATCTGGACCAGGACCTACCAGCCCACCTCCACCTGTACTTCACAGC
                S  V  G  P  E  S  G  P  G  P  T  S  P  P  P  P  V  L  H  S
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   361   CACATGACGTCCGGAAGTCCTTGCAAGGACTACATGACGTCATTTGGAAGCTACAGTCCC
                H  M  T  S  G  S  P  C  K  D  Y  M  T  S  F  G  S  Y  S  P
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   421   AATGGATCTTGCATGGGCTCTGGGGCTGCGGAGTTCCCTCCTATGACGTCATCCCACGTT
                N  G  S  C  M  G  S  G  A  A  E  F  P  P  M  T  S  S  H  V
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   481   CAAAGCCTGTCTCACATGTGCTCACCCTACGCATTTTGCCCCGATGACCCAGCAGCCTAT
                Q  S  L  S  H  M  C  S  P  Y  A  F  C  P  D  D  P  A  A  Y
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   541   TCGGGAAACTCATTCAAAGACCATCATCAGCAGACGGTTGTAGGGAGCCTCGATAAAAAG
                S  G  N  S  F  K  D  H  H  Q  Q  T  V  V  G  S  L  D  K  K
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   601   GATGTTCAAGGAAACTGTGTGACGGATGTAGATGACAAGTGCAAAATCGACAATAGCCAA
                D  V  Q  G  N  C  V  T  D  V  D  D  K  C  K  I  D  N  S  Q
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   661   ATTAGTACAACCACATCTACAGTGAGCTCATCTGGAGCGTCGTCAACCAATGCTGGATCT
                I  S  T  T  T  S  T  V  S  S  S  G  A  S  S  T  N  A  G  S
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   721   TCCCAAACTCTTTCTCACTCAAGGACCAAAAGAAAACCACGTGTTCTNTTCTCCCAGGCC
                S  Q  T  L  S  H  S  R  T  K  R  K  P  R  V  L  F  S  Q  A
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   781   CAAGTATACGAACTTGAACGTCGGTTCAAACAGCAACGTTATTTATCAGCTCCGGAGCGG
                Q  V  Y  E  L  E  R  R  F  K  Q  Q  R  Y  L  S  A  P  E  R
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   841   GAACACCTAGCACAGATGCTCAAACTCACCTCAACACAGGTAAAAATATGGTTCCAAAAT
                E  H  L  A  Q  M  L  K  L  T  S  T  Q  V  K  I  W  F  Q  N
               ....|....|....|....|....|....|....|....|....|....|....|....|
Gm tin   901   CGTCGTTATAAGTGCAAAAGGCAACGACAAGACAAGACATTAGAGATGAGTGCAGGGCTT
                R  R  Y  K  C  K  R  Q  R  Q  D  K  T  L  E  M  S  A  G  L
               ....|
Gm tin   961   CAACC 965
                Q
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Lebenslauf

Hilary L. Dove

geboren am:	03.05.1976 in Wien, Österreich
Familienstand:	ledig
Staatsangehörigkeit:	amerikanisch

Ausbildungsdaten

Schulausbildung:	1991 - 1994	Munich International School, München, Deutschland *Abschluss Mai 1994:* Bilinguales (Deutsch/Englisch) Internationales Baccalaeureat
Studium:	1994 - 1998	University of Pennsylvania, Philadelphia, USA *Abschluss 18. Mai 1998:* Masters of Arts (MA) in Biologie Bachelors of Arts (BA) in Biologie
	1998 - 1999	Universität zu Köln, Deutschland DAAD (Deutscher Akademischer Austauschdienst) Graduiertenstipendium für Forschung am Institut für Entwicklungsbiologie
Promotion:	Seit 2000	Universität zu Köln, Deutschland Doktorarbeit am Institut für Genetik bei Prof. Dr. Tautz.