Title: Molecular control of gut formation in the spider *Parasteatoda tepidariorum*

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ABSTRACT :

The development of a digestive system is an essential feature of bilaterians. Studies of the molecular control of gut formation in arthropods have been studied in detail in the fruit fly *Drosophila melanogaster.* However, little is known in other arthropods, especially in non-insect arthropods. To better understand the evolution of arthropod alimentary system, we investigate the molecular control of gut development in the spider *Parasteatoda tepidariorum (Pt)*, the primary chelicerate model species for developmental studies. Orthologs of the ectodermal genes *Pt-wingless (Pt-wg)* and *Pt-hedgehog (Pt-hh),* of the endodermal genes, *Pt-serpent (Pt-srp)* and *Pt-hepatocyte-nuclear factor-4 (Pt-hnf4)* and of the mesodermal gene *Pt-twist (Pt-twi)*areexpressed in the same germ layers during spider gut development as in *D. melanogaster*. Thus, our expression data suggest that the downstream molecular components involved in gut development in arthropods are conserved. However, *Pt-forkhead (Pt-fkh)* expression and function in spiders is considerably different from its *D. melanogaster* ortholog*. Pt-fkh* is expressed before gastrulation in a cell population that gives rise to endodermal and mesodermal precursors, suggesting a possible role for this factor in specification of both germ layers. To test this hypothesis, we knocked down *Pt-fkh* via RNA interference. *Pt-fkh* RNAi embryos not only fail to develop a proper gut, but also lack the mesodermal *Pt-twi* expressing cells. Thus, in spiders *Pt-fkh* specifies endodermal and mesodermal germ layers. We discuss the implications of these findings for the evolution and development of gut formation in Ecdysozoans .

Keywords:

endoderm, *Drosophila,* insect, *forkhead, hedgehog, hepatocyte-nuclear-factor-4,*

midgut, GATA, spider, Chelicerata

Introduction:

The evolution of multicellularity allowed organisms to generate specialized cell types, and lead to the emergence of an outer protective coat (the ectoderm) and an inner layer (the endoderm) involved in food absorption (Stainier, 2005). In most Bilateria, endoderm and mesoderm are commonly derived from a bipotential precursor cell or population of cells, called endomesoderm (Grapin–Botton and Constam, 2004). During gastrulation, the endoderm and mesoderm are internalized, and while endoderm gives rise to the epithelial lining of the gut, the mesodermal precursors will differentiate into connective tissue, coelom, somatic gonad, nephridia and most muscles. In all animal species studied so far, a portion of the gut is of ectodermal origin (Grapin–Botton and Constam, 2004).

Most studies of molecular aspects of gastrulation were initially performed in model organisms, either protostomes (*Drosophila melanogaster* and *Caenorhabditis elegans*) or deuterostomes (*Xenopus laevis, Danio rerio, Gallus gallus, Mus musculus*). The maternal factors involved in endoderm specification appear to be divergent among different animals, *e.g.* β-catenin, Nodal, VegT and Otx act in different deuterostomes providing initial cues for endoderm differentiation (reviewed in Grapin-Botton and Constam, 2007), while in *D. melanogaster* a complex maternal network involving the transmembrane receptor tyrosine kinase, Torso (Tor), is required for endoderm patterning. In contrast to this large variation in maternal control, a conserved set of genes are employed in the endomesodermal network of bilaterian digestive system differentiation (Annunziata et al., 2014; Boyle et al., 2014). *Forkhead* (FoxA -Fkh family), GATA factors and *hepatocyte nuclear factor 4* (*HNF4*), a highly conserved member of the steroid hormone receptor superfamily belong to this gene set. GATA factors and *fkh* expression are present in the endoderm even in non-Bilateria, such as the sea anemone (Cnidaria) *Nematostella vectensis* (Fritzenwanker et al., 2004; Martindale et al., 2004).

Molecular phylogenetic studies divided protostomes into Ecdysozoa (molting animals - Arthropoda and Nematoda, , among others) and Lophotrochozoa (Annelida, Mollusca, Platyhelminthes, among other groups) (Aguinaldo et al., 1997). Among Lophotrochozoa, Seaver and co-workers showed that FoxA, GATA and several other

transcription factors are expressed during gut development in the polychaete annelid, *Capitella* sp., suggesting that similar genes act during digestive system formation in Lophotrochozoa and other Bilateria (Boyle and Seaver, 2010; Boyle et al., 2014).

The two most widely used invertebrate model systems for developmental biology studies belong to the Ecdysozoa, the fruit fly *D. melanogaster* and the nematode *C. elegans.* Both species specify their germ layers before gastrulation, and display a series of adaptations regarding the type of embryogenesis and digestive system formation that they exhibit. Several of these characteristics deviate from the more ancestral type found in their phyla Arthropoda (*D. melanogaster*) and Nematoda (*C. elegans*) (Martin-Duran and Hejnol, 2015).

In *C. elegans,* endoderm precursors can already be identified at the 8-cell stage. At this stage a single blastomere displays Wnt activity, undergoes four rounds of division to generate a 16-cell intestinal primordium, which differentiates into a functional gut tube (Nance et al., 2005). This type of stereotypic embryonic development leads to a digestive system with a reduced cell number, a possible adaptation to the fast life cycle of this nematode (Martin-Duran and Hejnol, 2015).

The fruit fly *D. melanogaster* also shows a series of adaptations regarding early embryogenesis when compared to other arthropod eggs containing a larger amount of yolk. A typical derived characteristic of insect eggs is the establishment of the anteroposterior (AP) and dorsoventral (DV) axes during oogenesis. Thus, in the fly, a clear bilateral symmetry is evident without any trace of radial symmetry (reviewed in Roth and Lynch, 2009).

In *D. melanogaster*, the foregut, the hindgut and the Malpighian tubes originate from tube-like invaginations of ectodermal origin. Only the midgut has an endodermal origin; it is derived from cells in the anterior and posterior terminal regions in the early blastoderm, leading to the anterior and posterior midgut, respectively (reviewed in Yasugi and Mizuno, 2008, Hartenstein and Chipman, 2015, Figure 1A). The ectodermal components also form tubular structures that connect to the endodermal midgut, thus completing a continuous gut tube composed of the three major parts, the foregut, generated from the invagination of the stomodeum, the midgut, and the hindgut, generated from the proctodeum invagination (reviewed in Murakami et al., 1999, Lengyel and Iwaki, 2002). Genetic control of digestive system formation in *D.*

melanogaster is understood in some detail (Hartenstein and Chipman, 2015). Endoderm specification is under the maternal control of the terminal system, which provides positional cues in both the anterior and posterior terminal regions of the early embryo (Nusslein-Volhard et al., 1987; Nusslein-Volhard, 1991). Torso signaling is transduced via the Ras/Raf/MAPK cascade, and activates the two earliest zygotic gap genes *tailless* (*tll*) and *huckebein* (*hkb*), which are essential for gut development (Pignoni et al., 1990; Bronner and Jackle, 1991; Steingrimsson et al., 1991; Figure 1A). At least two groups of genes act downstream of *tll* and *hkb* to regulate gut formation in *D. melanogaster.* The first group contains *wingless* (*wg*) and *hedgehog (hh)*: two signaling molecules important for patterning the ectodermal components of the gut, the foregut and hindgut (Baker, 1988, Figure 1A). The second group specifies the endoderm. Three GATA factor genes (*srp, GATAd and GATAe*) and *HNF4* are essential for endoderm specification in *D. melanogaster* (Murakami et al., 2005; Okumura et al., 2005, Palanker et al., 2009; Zhong et al., 1993, Figure 1A). Posterior invagination is dependent on the maternal factor *caudal (cad)*, which activates *fkh* and the secreted protein Folded gastrulation (Fog). The T-Box transcription factor *Brachyenteron (Byn)* and the gap gene *Krüppel (Kr)* are also downstream targets of *Tll* and *Hkb* in the posterior region, and help to specify the hindgut and Malpighian tubules, respectively (reviewed in Hartenstein and Chipman, 2015; Yasugi and Mizuno, 2008). Lastly, *fkh* is essential for development of both the ectodermal and endodermal gut primordia (Weigel et al., 1990; Weigel et al., 1989). *fhk*, the *D. melanogaster* ortholog of FoxA family of transcription factors, is expressed during digestive system development in all metazoans investigated so far (Arenas-Mena, 2006; de-Leon, 2011; Fritzenwanker et al., 2004; Lee and Frasch, 2004).

All this knowledge of the molecular control of digestive system formation in *D. melanogaster* contrasts with scarce studies in other arthropods. In insects, studies have been restricted to the beetle *Tribolium castaneum* and the cricket *Gryllus bimaculatus* (Berns et al., 2008; Inoue et al., 2002). To study endoderm development in the amphipod crustacean *Parhyale hawaiensis*, cell lineage tracing was performed (Alwes et al., 2011).

Recent gene expression analysis in non-model ecdysozoans such as priapulids (Scallidophora) and onychophorans, provided important insights for the understanding

of digestive system formation (Janssen et al., 2015; Martin-Duran and Hejnol, 2015). The priapulid *Priapulus caudatus* shows holoblastic total cleavage, gastrulation by invagination and deuterostomic development of the mouth, which have been suggested as plesiomorphic features in Ecdyzosoa (Martin-Duran et al., 2012). Gene expression analysis of the foregut markers *NK2.1, foxQ2* and *FGF8/17/18*, midgut markers *GATA456* and *HNF4* and hindgut markers *wnt1* and *even-skipped (evx)* demonstrated that a conserved molecular patterning system is also present in Scallidophora, the third main ecdyzosoan lineage (Martin-Duran and Hejnol, 2015).

A recent study in onychophorans, the putative sister group of Arthropoda, also provided evidence that the expression of gut markers is conserved during mouth-anus development among Panarthropoda (Arthropoda plus Onychophora) (Janssen et al., 2015). *Euperipatoides kanangrensis (Ek) fkh* is expressed early in the germ disc and at later stages in the mouth and anus, while *Ek-caudal* is expressed in the anal valves, which arises from posterior ectoderm. Thus, at least regarding gut development, gene expression analysis in onychophorans suggests that there is a conserved molecular control in Panarthropoda. While molecular developmental studies regarding gut development have been performed in the aforementioned ecdysozoans; chelicerates, the putative sister group of Mandibulata (pancrustaceans and myriapods) (Regier et al., 2010), have been largely neglected. Among the 14 recognized orders, Araneae (spiders) are the most studied chelicerates regarding evolutionary developmental biology (Evo-Devo). The common house spider *Parasteatoda tepidariorum* (formerly *Achaearanea tepidariorum*) is considered an excellent chelicerate model system for Evo-Devo studies since several important techniques required for the analysis of gene expression and function (e.g. in situ hybridization, RNA interference) have been established in this species (Hilbrant et al., 2012; Schwager et al., 2015b).

Studies of *D. melanogaster* early development cannot be directly extended to other insects and non-insect arthropods (Hilbrant et al., 2012; Lynch and Roth, 2011). For instance, in the spider *P. tepidariorum* the egg is spherical without distinct axes during oogenesis (Schwager et al., 2015a). The AP axis of the spider embryo becomes evident in the course of the condensation mechanism during germ disc formation, and the DV axis is established by the migration of a distinct group of cells, the cumulus (Akiyama-Oda and Oda, 2006, 2010; Pechmann, 2016, Figure 1B). Thus, it is unlikely

that maternally deposited RNAs specify the gut anlagen during spider embryogenesis as in *D. melanogaster* and other insects. Second, *D. melanogaster* is a long-germ insect, where all segments are established almost simultaneously and the posterior ectodermal primordium invaginates early in development. In contrast, in short-germ arthropods such as the spider *P. tepidariorum*, the posterior segments are generated by a secondary process from a segment addition zone (SAZ) (reviewed in Hilbrant et al., 2012; Schwager et al., 2015b).

Molecular comparative studies with *P. tepidariorum* might also help to understand the long-standing question of the origin of the midgut epithelium in arthropod embryology (Roth, 2004 and references therein). Most of the issues relate to the role of yolk nuclei during midgut formation. Yolk nuclei are generated during syncytial stages, when most nuclei migrate to the periphery and generate the blastoderm. A few cells remain in the interior of the egg, the so-called primary vitellophages. At least in *D. melanogaster,* the yolk nuclei do not take part in embryonic or extraembryonic development. On the other hand, it is interesting to note that *srp* and later *fkh* are expressed in *D. melanogaster* yolk nuclei, which might represent a vestigial function of these cells (Reuter, 1994; Walker et al., 2000). Classical studies on basal insects, particularly apterygotes (Jura, 1972) suggest that the midgut arises from the yolk nuclei population. Histological studies in spiders during 19th and 20th centuries proposed that the yolk and extraembryonic cells give rise to the major part of the midgut epithelia and that the rectal sac and the Malphigian tubules arise from a distinct posterior rudiment (Balfour, 1880, Rempel, 1957, reviewed in Anderson, 1973). Kautzsch, 1910 was the first to distinguish the morphology of the posterior midgut rudiment and the proctodeum, while Holm suggested that the posterior midgut develops from posterior cumulus cells (Holm, 1952).

To bridge the gap of knowledge in the molecular control of digestive system formation in chelicerates, we cloned and analyzed the expression of several transcription factors and signaling molecules putatively important for gut formation in the spider *P. tepidariorum*. Our results show a conserved set of factors *(wg, fkh, hh)* being expressed in the proctodeum and stomodeum of this spider. *Pt-srp* and *Pt-hnf4,* two conserved endodermal genes, are expressed first in the extraembryonic yolk-rich cells and later in the middle region of the gut, the prospective midgut. We also carried

out knockdown of *fkh* in *P. tepidariorum*. *Pt-fkh* is the ortholog of the FoxA family, which is involved in digestive formation in all Bilateria (de-Leon, 2011), and also acts as a major regulator of gut development in *D. melanogaster* (Reuter, 1994; Weigel et al., 1989). *Pt-fkh* RNAi embryos do not develop a proper gut and, surprisingly, lack anterior mesodermal cells expressing *Pt-twist*. In summary, our analysis shows that a similar set of developmental genes are involved in gut specification in spiders and other arthropods.

Methods:

Animals: The spider culture was obtained from captured individuals in the surroundings of Cologne and maintained as previously described (Schwager et al., 2009). Embryonic staging is based on Mittmann and Wolff, 2012.

Ortholog gene identification and phylogenetic analysis: *Parasteatoda-forkhead- fkh* (**AB096073)** (Akiyama-Oda and Oda, 2003), *hedgehog - hh* (**AB125742)** (Akiyama-Oda and Oda, 2010)**,** *twist - twi* (**AB167807)** (Yamazaki et al., 2005), *wingless - wg* (**AB167808)** were cloned using PCR primers based on the known sequences of these genes. Degenerate primers were designed using the CODEHOP programme (Rose et al., 2003) to clone the following genes: *serpent (srp)* and *hepatocyte nuclear factor-4* (*hnf-4*). For *srp* a pair of degenerate primers Fwd- GARTGYGTNAAYTGYGGN and Rev - NCCRCANGCRTTRCANAC was used. For amplification of cDNA ends (RACE) using SMART cDNA kit (Clonetech) two primers were used for 5´-RACE GCATGCGGCCTTTATCATCGTATGAAC and 3´-RACE GACTGTCAGCCTCACGCCGTGTTG. For *hnf-4* a pair of degenerate primers Fwd-GCNACNGGNAARCAYTAYGG and Rev-TGY TSDATCATYTGCCANGTDAT was used. The following primers *Pt-hnf-4Fwd*-GCGACGGGAAAGCATTACGG and *Pt-hnf-4Rev*-ATCACTTGGCAAATGATCCAGCA were used for RACE as described above. The sequences of predicted genes (*Pt-hnf4* and *Pt-srp*) were used for phylogenetic analysis after the similarity was confirmed by reciprocal BLAST searches and a Conserved Domain search on CDD (Marchler-Bauer and Bryant, 2004). The putative *P. tepidariorum* protein sequences were aligned with ClustalW (http://www.ebi.ac.uk/clustalw) to several family members known in other taxa. Regions of the alignment with gaps in most sequences were omitted from phylogenetic analysis by masking them in Seaview (Galtier et al., 1996). The most informative amino acid substitution model was calculated with Prottest (Abascal et al., 2005). Maximum likelihood phylogenies were generated with PhyML (Guindon et al., 2005). Trees were edited in MEGA7 (Kumar et al., 2016). For GATA phylogeny, sequences were retrieved from Gillis et al., 2008. GeneBank accession numbers are available in the supplemental data. BLAST searches were also performed in a recently published *P. tepidariorum* embryonic transcriptome (Posnien et al., 2014) and in the genome (Schwager et al., 2017).

In situ **hybridization and knockdown via parental RNAi (pRNAi):** Colorimetric *in situ* hybridization for the aforementioned genes was performed as previously described (Akiyama-Oda and Oda, 2003; Damen and Tautz, 1998; Prpic et al., 2008b). For fluorescent in situ hybridization Fast Red TR/Naphtol AS-MX Alkaline Phosphatase Substrate Tablets Set (SIGMA - F4648) was used and images were obtained with a LSM 700 confocal microscope (Zeiss). Parental RNAi was performed as previously described (Akiyama-Oda and Oda, 2006). We used dsRNA against two non-overlapping fragments of *Pt-fkh.* Based on the published *Pt-fkh* mRNA sequence (Akiyama-Oda and Oda, 2003) two primer sets were designed to amplify regions between 70-720 and 860-1520 base pairs of the coding region . Both dsRNAs were injected into female spiders and gave identical phenotypes. As a control, GFP dsRNA was injected at the same concentration as *Pt-fkh* dsRNA. Nuclear DAPI staining (4',6-diamidino-2-phenylindole) was performed for 10 minutes by applying a solution of 1 µg/µl in PBST. Imaging was performed using a LEICA M165 stereoscope with a UV filter.

Cross sections of spider embryos: After *in situ* hybridization the stained embryos were dehydrated in ethanol, transferred to acetone and embedded in Durcupan (Fluka) according to manufacturer´s instructions. Semi-thin sections (7µm) were obtained with a Leica microtome (Model RM2235) and photographed using an Axiovision (Zeiss) microscope as previously described (McGregor et al., 2008).

Cell death assay: The cell death assay was performed according to Prpic et al., 2008a.

Results:

Orthologs of hedgehog, forkhead and wingless are expressed in the presumptive gut ectoderm during spider embryogenesis

In *D. melanogaster, forkhead* (*fkh), wingless (wg)* and *hedgehog (hh)* are expressed during stomodeum and proctodeum formation (Figure 1, reviewed in Hartenstein and Chipman, 2015), therefore, we cloned and analyzed the expression of their respective orthologs in the spider *P. tepidariorum.*

In *P. tepidariorum*, *Pt-fkh* displays a complex expression pattern during early embryogenesis (Akiyama-Oda and Oda, 2003; Oda et al., 2007). At stage 3, *Pt-fkh* is detected at the primary thickening (pt) (Akiyama-Oda and Oda, 2003, Oda et al., 2007, Sup. Figure 1A). At stage 4, besides its expression in the pt, it is also observed at the rim of the germ disc (Sup. Picture 1B), and in the presumptive extraembryonic cells (Sup. Figure 1B). At stage 5 *Pt-fkh* expression is observed in the central endodermal cells (cEND) of the cumulus and at the peripheral endodermal cells (pEND), located at the rim of the germ disc (Akiyama-Oda and Oda, 2003, Oda et al., 2007, Sup. Figure 1C,D). During stage 5, cumulus mesenchymal cells (CM) migrate towards the rim of the germ disc and mediate the breaking of radial symmetry (Akiyama-Oda and Oda, 2006, Sup. Figure 1E,F). Close to the end of cortical migration, the cumulus starts to disappear. This area marks the prospective dorsal region, whereas the center of the germ-disc represents the posterior pole (Akiyama-Oda and Oda, 2006; Mittmann and Wolff, 2012). At stage 8, when the germ band has formed, *Pt-fkh* is still detected in the extraembryonic cells (Sup. Figure 1G) and at the most anterior region of the head, possibly the future stomodeum (Figure 2A,H). At stage 9 the ventral midline expression is clearly evident (Figure 2B,I). but disappears by, stage 11 (Figure 2J) However longitudinal cross sections show *Pt-fkh* expression in an anterior region, corresponding to the posterior end of the stomodeum (st) and in the brain primordium (br) at stage 11 (Figure 2D,K). At the posterior region *Pt-fkh* is detected at the outer ectodermal layer of the segment addition zone (SAZ), the prospective proctodeum (Figure 2D,L - proctodeum - pr) and at stage 12 *Pt-fkh* expression is restricted to these two regions (Figure 2E,M). Shortly before hatching (stage 14-Figure 2F,N), sagittal sections show

specific expression at the stomodeum (st), the proctodeum (pr) and the brain primordium (br) (Figure 2G,O,P). The expression of *Pt-fkh* suggests this gene plays a role not only in proctodeum and stomodeum (ectoderm) specification but also in early events of embryogenesis (Akiyama-Oda and Oda, 2003).

hh is known to be important for stomodeum and proctodeum formation in vertebrates and arthropods (Hoch and Pankratz, 1996; Parkin et al., 2009; Simonnet et al., 2004). *Pt-hh* expression has been previously described in detail during early stages of *P. tepidariorum* (Akiyama-Oda and Oda, 2010), but expression of this gene during later stages was not reported. During stages 8 and 9, *Pt-hh* is expressed as a segment polarity gene and also in the proctodeum and stomodeum (Figure 3A,H,B,I,C,J). Sagittal cross sections at stage 11 show *Pt-hh* expression at the posterior region of the stomodeum (st) (Figure 3D,K), at the posterior region of the leg bearing segments and in two domains at the SAZ i (Figure 3D,L). One expression domain is segmental and of ectodermal identity (Figure 3L) and the other corresponds to the future hindgut (Figure 3L). This expression pattern is very similar at stage 12 (Figure 3E,M). Shortly before hatching at stage 14, *Pt-hh* displays a more restricted pattern at the posterior region of the leg bearing segments (Figure 3F,N) and sagittal cross sections show expression at the prospective stomodeum, in the brain (br) (Figure 3G,O), and in the posterior region of the prospective hindgut (Figure 3G,P).

The *wingless* (*wg*) gene is involved in foregut and hindgut formation in *D. melanogaster* (Hoch and Pankratz, 1996). In *P. tepidariorum*, at stage 9, *Pt-wg* expression is only segmental (Sup. Figure 2A,E), but at stage 11 it is observed at the posterior region after segment generation has ended (Sup. Figure 2B,F). Longitudinal cross sections reveal an expression domain in the outer (ectodermal) part of the posterior region, (Sup. Figure 2G,H). At stage 14 the expression in each appendage and in the ventral region is still observed(Sup. Figure 2C,D). *Pt-wg* does not appear to be expressed at the stomodeum. Taken together, expression analysis of *Pt-hh* and *Pt-wg* suggests a possible role of these genes in proctodeum specification, *Pt-hh* and *Pt-fkh* in stomodeum development, while *Pt-fkh* might also play a role during early development, possibly in germ layer specification.

Orthologs of hnf-4 and srp are expressed in the midgut during spider embryogenesis

Hepatocyte nuclear factor 4 (*hnf-4*) is required for endodermal patterning in all animals investigated so far, therefore we cloned an *hnf-4* ortholog in *P. tepidariorum* using degenerate primers (Sup. Figure 3A). *Pt-hnf4* displays typical domains found in other family members including a DNA binding domain and a ligand binding domain at the C-terminus (Sup. Figure 3B, Sladek, 2011). Phylogenetic analysis shows that *Pt-hnf4* groups together with other family members among arthropods and other metazoans (Sup. Figure 3A). BLAST searches of the *P. tepidariorum* genome (Schwager et al., 2017) identified a single ortholog of *Pt-hnf-4*.

In *P. tepidariorum*, *Pt-hnf4* expression starts at stage 8 in the prospective extraembryonic cells (Figure 4A,H), and these cells still express *Pt-hnf4* at stage 11 (Figure 4B,I). During germ band inversion, at stage 12, these extraembryonic *Pt-hnf-4* expressing cells start to be enclosed by embryonic cells (Figure 4C,J). Between stages 13 and 14, when dorsal and ventral closure take place, most expression of *Pt-hnf-4* is detected in the opisthosoma (Figure 4D,E,K,L). To determine if *Pt-hnf-4* is expressed in the endodermal tissue, sagittal cross sections were performed (Figure 4F,G). These sections showed *Pt-hnf-4* expression in the inner cell layer, along the midgut (Figure 4G). Interestingly, *Pt-hnf4* expression extends to the anterior midgut (Figure 4M), close to the stomodeum (Figure 4M) and to the posterior midgut (Figure 4N), close to the proctodeum (Figure 4N). Altogether, *Pt-hnf-4* is first expressed in the extraembryonic cells and later in the endoderm (midgut) of the gut.

To investigate if other conserved endodermal genes are involved in spider gut formation, we cloned GATA transcription factors using degenerate primers (see methods). Additional BLAST searches into the *P. tepidariorum* embryonic transcriptome (Posnien et al., 2014) and genome (Schwager et al., 2017) revealed seven GATA genes (Supplementary Table). Two genes are truncated and were not included in the phylogenetic analysis (XP_015919717.1 and XP_015907840.1). Three genes were classified as *Ptep-GATAlikeA* (XP_015929398.1), *Ptep-GATAlikeB* (XP_015907843.1) and *Ptep-GATAlikeC* (XP 015918057.1), since it was not clear from the phylogenetic analysis if they are orthologs of *pannier (pnr)* or *grain (grn).* A clear

ortholog of *Pt-pnr* (XP_015929399.1) was also distinguished. Importantly, we detected only a single ortholog of *serpent (srp)* (XP_015929889.1), an important GATA factor for endoderm development in *D. melanogaster* (Campbell et al., 2011) (Sup. Figure 3C). *Pt-srp* expression starts at stage 8 in the presumptive extraembryonic cells (Figure 5A,G) and is maintained in this cell population at least until stage 11 when the spider embryo has completed segment generation from the SAZ (Figure 5B,H). At stage 14 these putative extraembryonic cells expressing *Pt-srp* might be incorporated into the embryo, mainly into the opisthosoma (Figure 5C,D,I,K).

To visualize the exact location of *Pt-srp* expressing cells at stage 14, we performed sagittal cross sections. As previously observed for *Pt-hnf4*, *Pt-srp* is expressed along the midgut (mg- Figure 5E,L), which forms a continuous tube surrounding the yolk mass (Figure 5E,L). However, in contrast to *Pt-hnf4*, *Pt-srp* expression does not extend towards the anterior part of midgut close to the invaginating stomodeum (Figure 5F). In the posterior region, *Pt-srp* expression is more restricted than *Pt-hnf4* (Figure 5M). While *Pt-hnf4* is found throughout the inner cell mass of the posterior region (Figure 4G,N), *Pt-srp* is located in a single cell layer of the midgut beneath ectoderm (Figure 5M). Altogether, *Pt-srp* and *Pt-hnf-4* demarcate the prospective midgut during spider embryogenesis suggesting a role in endodermal patterning (Figures 4 and 5). In addition, both genes are expressed first in the prospective extraembryonic cells and later in the midgut, suggesting that either these cells migrate to form the gut or that those genes are switched on in two different cell populations during embryonic development.

Pt-twist is expressed along the mesoderm involved in gut establishment

The association of mesodermal with the endodermal tissue and mesenchymal-epithelial interactions are necessary for gut formation (Wu et al 2007). Mesoderm specification and commitment in *D. melanogaster* is dependent on the action of the bHLH transcription factor *twist*.(Thisse et al., 1987). In *P. tepidariorum, Pt-twist (Pt-twi)* has been previously identified (Yamazaki et al 2005) and its expression has been reported during early stages. Here we focus on the analysis of *Pt-twi* expression during

the embryonic stages important for gut formation. At stage 8 during germ band elongation *Pt-twi* is expressed in the mesoderm in a segmental fashion (Yamazaki et al 2005, Figure 6A). Shortly afterwards, at stage 9, mesodermal segmental expression has largely disappeared and *Pt-twi* mRNA is more restricted to the region surrounding the stomodeum (Figure 6B). At stage 11 *Pt-twi* expression also appears in the posterior region, flanking the proctodeum (Figure 6C). At stage 14 *Pt-twi* expression is only observed in these two domains at mesenchyme flanking bilaterally the stomodeum and proctodeum (Figure 6D,E). Transversal cross sections show *Pt-twi* in the mesenchymal (me) cells surrounding the stomodeum (st) (Figure 6G), and in the posterior cells surrounding the proctodeum (Figure 6F).

Pt-fkh* is required for early mesoderm specification and stomodeum formation in *P. tepidariorum

fkh (*FoxA*) transcription factors are expressed in endodermal or endomesodermal precursors in several metazoan lineages (Arenas-Mena, 2006; Azzaria et al., 1996; de-Leon, 2011; Fritzenwanker et al., 2004; Janssen et al., 2015; Weigel et al., 1989). Together with GATA and HNF4 transcription factors they constitute the most conserved factors required for endoderm development (Grapin-Botton and Constam, 2007).

To test if *Pt-fkh* is important for gut patterning in the spider *P. tepidariorum*, we performed pRNAi against *fkh.* Fluorescent in situ hybridization (FISH) showed *Pt-fkh* knockdown was effective, only nuclear transcripts are observed after *Pt-fkh* RNAi when compared to the cytoplasmic expression of control embryos (Sup Fig. 4).

Pt-fkh RNAi embryos display severe defects during early embryogenesis and do not hatch, with embryonic development arresting at stage 11. *Pt-fkh* function was first analyzed by assaying *Pt-hh* expression in control and *Pt-fkh* RNAi embryos (Figure 7). Pt-*fkh* RNAi embryos do not display alterations of the segmentation pattern*, i.e.* the number of *Pt-hh* stripes does not change, although these segments show an irregular shape upon DAPI staining (Figure 7A,D). Further analysis of *Pt-hh* expression at stage 8

and 9 indicated that stomodeum formation was impaired in *Pt-fkh* RNAi embryos, suggesting an important role of *Pt-fkh* in anterior gut patterning (Figure 7B,C,E,F). The stomodeum was completely absent in strong *Pt-fkh* RNAi embryos (Figure 7E); cells which should form the stomodeum apparently fail to migrate and stay spread along the extraembryonic region close to the head (Figure 7E). Importantly, some *Pt-fkh* RNAi embryos that were more weakly affected had a smaller stomodeum when compared to controls (Figure 7F). Our results suggest that *Pt-fkh* is required for stomodeum formation, the anteriormost ectodermal fate of the gut.

We also analyzed the role of *Pt-fkh* in the specification of the mesoderm and endoderm. Expression of the mesodermal marker *Pt-twist (Pt-twi)* largely disappeared after *Pt-fkh* RNAi (Figure 8A,B), and only the most posterior prosomal segments still display *Pt-twi* (Figure 8B) at stage 8. Analysis of sagittal cross sections shows that a few cells below the ectoderm do exist in *Pt-fkh* RNAi embryos, but these cells do not express *Pt-twi* (Figure 8E,E´,F,F´). Lack of mesodermal *Pt-twi* expressing cells was already evident at early and mid stage 6 (Sup. Figure 5), further supporting that *Pt-fkh* is required for early *Pt-twi* expression and consequently mesoderm specification at the anterior region of the embryo.

Expression of the endodermal marker *Pt-srp* was also affected after *Pt-fkh* RNAi. While in control embryos, *Pt-srp* is expressed in an organized group of presumptive extraembryonic cells during at stage 8 (Figure 8C), *Pt-fkh* RNAi embryos show a disorganized arrangement of extraembryonic cells, which express *Pt-srp* (Figure 8D - arrow). Detailed sagittal cross sections in control embryos at stage 8 show *Pt-srp* expression only at the dorsal extraembryonic cells (Figure 8G,G´). In contrast, *Pt-fkh* RNAi embryos display an ectopic distribution of *Pt-srp* expressing cells, including scattered cells underneath the germ band (Figure 8H,H´ - arrow). At stage 11 these *Pt-fkh* RNAi embryos die, preventing an extended analysis of its role during gut development. Taken together, our results show that *Pt-fkh* is an important gene for stomodeum formation and early activation of the mesodermal gene *Pt-twi.*

Discussion:

Gut formation during embryogenesis in Eumetazoans follows a series of morphological events that have been well characterized in several species (Stainier, 2002, Arendt et al., 2001; Rubin, 2007). In arthropods the molecular control of these events has only been studied in detail in the insect *D. melanogaster* (reviewed in Yasugi and Mizuno, 2008 and Hartenstein and Chipman, 2015). Here, we have investigated the molecular control of gut formation in a non-insect arthropod, the spider *P. tepidariorum*. Our study provides a first attempt to investigate the molecular aspects of gut development in chelicerates, the sister group of the mandibulates.

Evolution of foregut and hindgut patterning – wg *and* hh *expression in the proctodeum and stomodeum*

As well as the endoderm, the ectoderm is also important for patterning the anteriormost region of the embryo, the stomodeum, which will give rise to the foregut, and the posteriormost region, the proctodeum, which will give rise to the hindgut. In *D. melanogaster*, the foregut arises by invagination of the stomodeum from ectoderm on the ventral midline of the intercalary segment and the hindgut arises slightly later by invagination of the proctodeum. Only later in development both stomodeum and proctodeum communicate with the lumen of the midgut (Skaer, 1993). Several pathways important for the establishment of proctodeum and stomodeum have been described in *D. melanogaster* including *hh, dpp* and *wnt* (reviewed in Yasugi and Mizuno, 2008). A similar situation occurs in the flour beetle *Tribolium castaneum,* where *hh* and *wg* are expressed during segmentation as well as in the foregut and hindgut. However, functional analysis of *hh* and *wg* function during gut formation in *T. castaneum* is masked by a segmentation phenotype (Bolognesi et al., 2008a; Bolognesi et al., 2008b; Farzana and Brown, 2008). In the hemimetabolous insect *Gryllus bimaculatus hh* and *wg* are expressed in both the foregut and hindgut (Inoue et al., 2002), suggesting a conserved role of these genes in insects. Fate mapping studies in the amphipod crustacean *P. hawaiensis* showed that the endoderm is generated from a single micromere distinguished at the eight cell-stage (Gerberding et al., 2002; Nast

and Extavour, 2014). This finding highlights the plasticity of the system in a holoblastic (total) cleavage arthropod. In onychophorans, the putative sister group of arthropods, *Wnt1/Wg* is expressed at the proctodeum (Eriksson and Tait, 2012), a similar posterior expression (hindgut) of *wnt1/wg* was also observed in priapulids (Martin-Duran and Hejnol, 2015) and during larval stages of the annelid Capitella sp. in the anus (Seaver and Kaneshige, 2006). Thus, posterior expression of Wnt1/Wg during hindgut development is conserved in all Bilateria investigated so far including the data presented here for the spider *P. tepidariorum*. Thus, *hh* expression during gut development seems to be conserved in Bilateria (Kang et al., 2003).

We show here that in *P. tepidariorum, hh* and *wg* are expressed in the stomodeum and proctodeum during embryogenesis, suggesting a conserved role of these signaling pathways in the ectodermal component of the gut in arthropods (Figure 3 and Sup. Figure 2). Similarly, in vertebrates, Hh signalling from the brain primordium is required for proper specification of the stomodeum (Eberhart et al., 2006).

Divergent aspects of gut formation between flies and spiders - maternal versus zygotic input

In *D. melanogaster* the anterior and posterior endoderm are specified maternally by activity of the terminal system (Ambrosio et al., 1989; Bronner and Jackle, 1991; Casanova and Struhl, 1993). This system seems conserved among beetles and flies (Schoppmeier and Schroder, 2005). Torso becomes activated specifically at the anterior and posterior poles by a ligand - presumably a cleaved form of the Trunk protein - which diffuses locally from a source near the poles. Upon activation, Torso triggers the Ras/Raf/MAPK pathway, which will ultimately lead to expression of the zygotic genes *tll* and (*hkb*), initiating the developmental programs that give rise to the anterior-most and posterior-most terminal regions of the embryo. Interestingly Torso and Trunk are absent in honeybee genome (Dearden et al., 2006), but the role of *tll* in the posterior region appears conserved in insects (Wilson and Dearden, 2009). Recent analysis in insects showed that the canonical terminal system is an evolutionary innovation of holometabolous insects (Duncan et al., 2013). In *T. castaneum* the Torso

pathway is detected at both poles but *Tc-tll* is observed only at the posterior region of the egg (Schoppmeier and Schroder, 2005; Schroder et al., 2000). Analysis of the role of *Tc-tll* in endoderm formation is problematic because the segmentation phenotype temporally precedes gut formation.

Since spider embryos are radially symmetric until cumulus migration (Oda and Akiyama-Oda, 2008), and an anterior-posterior axis seems not to be present before germ-disc condensation (Pechmann 2016), a maternal determination of the endoderm in both poles in spiders is less likely. Cumulus migration seems to be zygotically regulated by *hh* signalling (Akiyama-Oda and Oda, 2010). Dynamic expression of *hh* and *otd* is essential for anterior-posterior patterning, particularly setting up the anterior segments (Kanayama et al., 2011; Pechmann et al., 2009). In contrast to flies, it is therefore unlikely that anterior or posterior specification of the endoderm and ectoderm occurs during spider oogenesis. Thus, gut patterning must instead occur via zygotic gene interactions and germ layer specification during germ disc and cumulus formation. Since *fkh* is expressed during digestive system development in Bilateria (Akiyama-Oda and Oda, 2003; Arenas-Mena, 2006; de-Leon, 2011; Fritzenwanker et al., 2004; Janssen et al., 2015; Schroder et al., 2000; Weigel et al., 1989), we investigated its function during spider gut development (see below).

- Pt-fkh*'s role in* twist *regulation: a possible endomesodermal population in spiders*

A major finding of this study is that *fkh in P. tepidariorum* is not only important for the formation of the stomodeum (Figure 7), but also for the expression of the mesodermal gene *Pt-twi* (Figure 8, Sup.Figure 5). This represents a deviation from the *D. melanogaster* paradigm, since in this dipteran *twi* expression is maternally activated at the ventral region under the control of Dorsal, a NF-Kβ transcription factor (Jiang et al., 1991). *twi* is part of the gene regulatory network involved in dorsoventral patterning not only in *D. melanogaster* (e.g. Zeitlinger et al., 2007), but also in other insects (Pers et al., 2016; Stappert et al., 2016). Insect eggs display an extreme case of anticipation of bilateral symmetry (see introduction), thus the germ layers, including endoderm and mesoderm, are largely specified early in *D. melanogaster*, before gastrulation. While the mesoderm is under DV axis control, in insects the endoderm is

controlled by the terminal system (AP patterning), at least in *D. melanogaster*. In contrast, *P. tepidariorum* eggs are radially symmetric when laid. Abipotent precursor of endoderm and mesoderm under *Pt-fkh* control might exist, since anterior *Pt-twi* mesodermal expression is dependent on *fkh* (Figure 8A,B, Sup Picture 5).

Further support for the existence of bipotent endomesodermal precursors during spider embryonic development is corroborated by previous studies. Oda et al., 2007 identified three cell populations in spider embryos which express *Pt-fkh* at stage 5: the cumulus cells (CM); mesodermal (pMES) and peripheral endodermal (pEND) precursors at the rim of the germ disc (Akiyama-Oda and Oda, 2003, Sup. Figure 1). *Pt-delta* RNAi embryos lack prosomal expression of *Pt-twi* and *Pt-fkh* (Oda et al., 2007). Since *Pt-fkh* RNAi embryos analyzed here lack *Pt-twi* expression at the anterior region (Figure 8) it is possible that *Pt-fkh* induces the mesodermal germ layer via *Pt-twi* expression. Our data is also in agreement with Akiyama-Oda and Oda, 2016, which recently showed by double-fluorescent *in situ* hybridization that *Pt-fkh*-positive cells at stage 5 are subdivided into *Pt-fkh*-positive cells (endodermal precursors) and *Pt-fkh* plus *Pt-twi*-positive cells (mesodermal precursors). Thus, *Pt-fkh* is involved in *Pt-twi* activation, an essential gene for mesoderm patterning, a characteristic so far only described in spider embryos among arthropods (Figure 9). The connection between *fkh* expression and endomesodermal germ layer precursors has been described in other ecdyzosoans such as nematodes (Nance et al., 2005) and priapulids (Martin-Duran and Hejnol, 2015), in annelids, representing lophotrochozoans (Boyle et al., 2014), and also in several deuterostomes (reviewed in de-Leon, 2011).

Analysis of *Pt-fkh* RNAi also suggests a conserved role of *forkhead* transcription factor in stomodeum formation (Figure 7). *Drosophila fkh* is also expressed in the entire region of the ectodermal gut structure, and is essential for development of the foregut, the hindgut and for endoderm development (Hoch and Pankratz, 1996; Weigel et al., 1989). Similarly, *fkh* acts during hindgut formation in the beetle *T. castaneum* (Schoppmeier and Schroder, 2005; Schroder et al., 2000). Unfortunately, *Pt-fkh* RNAi lead to premature embryonic lethality, precluding the analysis of midgut and hindgut formation, which occurs at later developmental stages. Thus, *fkh* functional analysis in *P. tepidariorum* presented here highlights the importance of a comparative

evolutionary approach in order to expand the knowledge of gut molecular control in non-insect arthropods.

Conserved aspects of midgut formation in metazoans – transcription factors in the endoderm

Our results suggest that the downstream zygotic components involved in endoderm patterning are conserved in arthropods. *Pt-hnf-4* is expressed in the "extraembryonic" cells at stage 8 (Figure 4A,H), which are later observed into the midgut of the embryo (Figure 4C-G). A similar expression pattern was observed for the GATA factor *srp* during embryogenesis (Figure 5). The major difference in *Pt-hnf4* and *Pt-srp* expression is observed in the most posterior embryonic region at stage 14. *Pt-hnf-4* is expressed in the internal mesenchymal cells, while *Pt-srp* is detected in the cells beneath the ectoderm. Both genes are expressed in the endodermal tube (Figure 4N and 5M). It is possible that those extraembryonic cells are incorporated into the embryo and contribute to gut formation after segment formation has ended. Although cell death occurs in the extraembryonic cell population (Sup. Figure 6) massive cell death is not observed until the gut is established, suggesting that a network of "extraembryonic" cells expressing endodermal markers might be involved in yolk consumption and gut formation in spiders.

GATA factors (*srp, GATAd and GATAe*) and *HNF4* are important for endoderm specification in the insect *D. melanogaster* (Murakami et al., 2005; Okumura et al., 2005). Expression of GATA and *HNF4* has also been reported in several other Ecdyzosoa such as the priapulid *P. caudatus* (Martin-Duran and Hejnol, 2015), in the lophotrochozoan polycheate *C. teleta* (Boyle et al., 2014) and even in deuterostomes such as sea urchins (Annunziata et al., 2014). Thus the downstream factors responsible for endoderm differentiation seem to be conserved in bilaterians. To conclude, our expression analysis in spiders corroborates the hypothesis of an evolutionary conserved mechanism, (i.e. cooperation between HNF3/Fkh and GATA factors) for endoderm development in Eumetazoans (Grapin-Botton and Constam, 2007).

Evolution of vitellophage function and yolk consumption during embryogenesis in arthropods

The origin of the midgut in arthropods has been a mystery since the beginning of the last century (Johannsen and Butt, 1941, reviewed in Roth 2004, Anderson, 1973 see introduction). A key issue in midgut development is the role of the large polygonal yolky cells, which are generated during embryogenesis of several arthropods. Rounded eggs rich in yolk are characteristic of most chelicerates. Only mites, pseudoscorpions and viviparous scorpions produce small eggs showing evidence of a secondary reduction in yolk (Anderson, 1973; Schwager et al., 2015b). The sister group of euchelicerates, pycnogonids, display total and equal cleavages that are irregular (Ungerer and Scholtz, 2009). Classical studies in Xiphosura (horseshoe crabs) (reviewed in Anderson, 1973) and recent fluorescent injection studies in *P. tepidariorum* (Kanayama et al., 2010) show that early cleavages in these groups are superficial (i.e. without membrane formation between energids and in the centre of the egg). This superficial cleavage mode has been considered the ancestral type among chelicerates (reviewed in Anderson, 1973; Schwager et al., 2015b). After the 16-cell stage, at least in *P. tepidariorum,* cell proliferation takes place, leading to the formation of a contiguous blastoderm at stage 2. A condensation-like mechanism involving cell shape changes between stage 2 and 4 generates the germ disc and the large extraembryonic cells (Pechmann, 2016).

Here we provide evidence that *Pt-fkh, Pt-hnf-4* and *Pt-srp* are expressed in the large extraembryonic cells in an organized fashion after gastrulation. These extraembryonic cells surround the yolk. It is possible that these extraembryonic cells play an important function in yolk digestion, perhaps as a primary digestion system. This hypothesis is corroborated by the analysis of *Pt-fkh* RNAi embryos, which display disorganized extraembryonic cells (Figure 8). These extraembryonic cells that express *Pt-srp* and *Pt-hnf4* might be incorporated into the midgut of wild-type embryos (Figure 4 and Figure 5). In the future, knockdown of *Pt-hnf4* and *Pt-srp* in *P. tepidariorum* might unveil if these genes are important in extraembryonic cells and later during midgut formation. Therefore, while classical histological studies (Balfour, 1880; Holm, 1952; Kautzsch, 1910) and the molecular data presented here favor an ancestral role of these “extraembryonic” cells for midgut formation in non-insect arthropods, only

detailed fate-map studies will be able to unveil the respective contribution of each cell group to gut formation.

Conclusions:

Taken together our results show that the expression of downstream orthologs involved in gut formation in insects is conserved in spiders, and by extension, in non-insect arthropods. In contrast, the upstream maternal terminal system is likely to be more derived as previously suggested for hemimetabolous insects (Duncan et al., 2013). The *Pt-fkh* gene is required not only for gut specification as in *D. melanogaster*, but also for anterior mesoderm establishment. Our results are compatible with the existence of a bipotent precursor of endoderm and mesoderm in spiders. Finally, our data further establishes the spider *P. tepidariorum* as an emerging model system for chelicerate research (Hilbrant et al., 2012, Oda and Akiyama-Oda, 2008, Schwager et al., 2015b) which will enable further studies on the evolution of gut formation in arthropods.

Figure Legends:

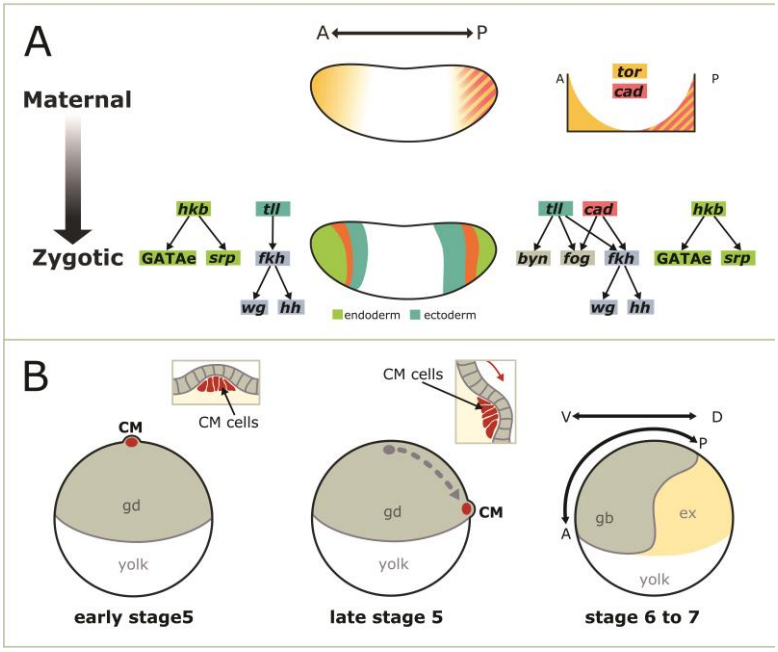

Figure 1: Early fate map of the long-germ insect *Drosophila melanogaster* and of the short-germ spider *Parasteatoda tepidariorum*. (A) In *D. melanogaster* the maternal control of gut development is initiated by the graded distribution of Torso active ligand, a C-terminal fragment of the Trunk protein, which leads to high activity at both anterior and posterior regions of the egg. At the posterior region, maternal Caudal (cad) is also required for patterning. Zygotic patterning is achieved by the interaction of transcription factors and signaling molecules, which are different anteriorly and posteriorly *e.g.* Folded gastrulation (Fog) and Brachyenteron (Byn) are only required at the posterior (Adapted from Hartenstein and Chipman, 2015; Yasugi and Mizuno, 2008). Anteriorly, *fkh* is under the control of *tll*, while posteriorly is controlled by *tll* and *cad*. Ectodermal and endodermal regions are labelled by different colors, blue and green respectively. Orange color demarcates the proventriculus, at the anterior region, and Malpighian tubules at the posterior region. (B) In the symmetrical spider egg of *P. tepidariorum* maternal patterning system is not evident during early stages. A symmetry breaking event is observed during stage 5 when cumulus mesenchymal (CM) cells migrate laterally beneath the ectoderm. CM migration and its secreted signaling molecules leads to the formation of the extraembryonic cells (ex). At stage 7 a segmented germ band (gb) with clear AP and DV polarity becomes evident. gd - germ disc. CM - cumulus mesenchymal cells (adapted from Akiyama and Oda, 2010).

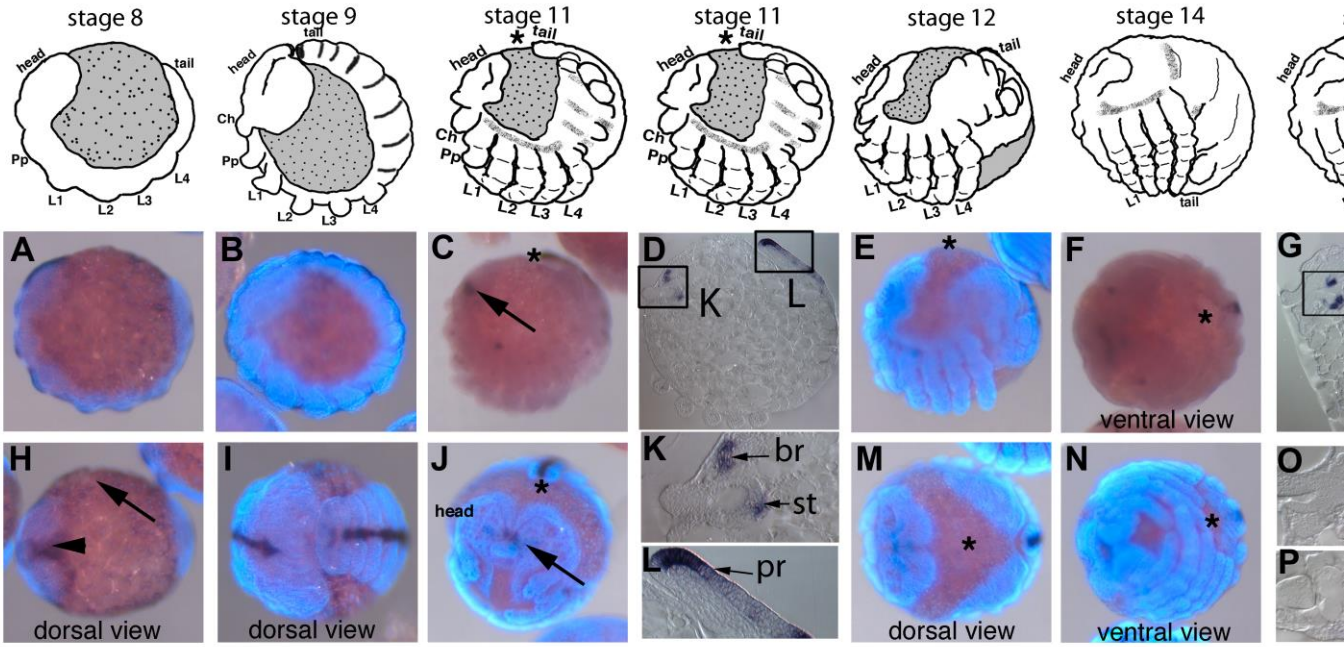

Figure 2: *Pt-forkhead* (*Pt-fkh*) is expressed in the ventral midline, stomodeum and proctodeum. The top row of panels displays schematic drawings of the images of the embryos below. (A,B,C,E) Anterior is to the left and lateral views are shown in all panels. DAPI counterstainings (light blue) highlight the embryo in contrast to the yolk, with the exception of C and F. (D,K,L,G,O,P) are longitudinal cross sections. (A,H) At stage 8 *Pt-fkh* is expressed in the presumptive extra-embryonic cells (arrow), in the tissue surrounding the head (arrowhead) and at the ventral midline (not shown). (B,I) At stage 9, *Pt-fkh* is detected at the ventral midline. (C,J) At stage 11, *Pt-fkh* expression is observed in the stomodeum (arrow) and in the posterior region of the germ band (asterisk). (D) Longitudinal cross section of stage 11 embryos show *Pt-fkh* expression at the most posterior cells of the stomodeum (BOX-K) and at the epithelial cells of the posterior region (BOX-L). (K) Higher magnification shows *Pt-fkh* expression at the stomodeum (st) and in the brain (br). (L) At the posterior region of the embryo *Pt-fkh* is expressed at the ectodermal cells of the posterior region (pr). (E,M) At stage 12, cells from the posterior region start to migrate towards the ventral side. An asterisk marks the dorsal side of the embryo. (F,N). At stage 14, when the posterior region (asterisk) reaches the ventral side, *Pt-fkh* expression is detected only at the anterior and posterior regions. (G,O,P) Longitudinal cross section of stage 14 embryos highlighting *Pt-fkh* expression at the anterior (Box-O) and at the posterior (Box-P) embryonic regions. (O) Higher magnification of the anterior embryonic region shows *Pt-fkh* expression in the posterior region of the stomodeum (st) and in the brain (br). (P) Higher magnification of the posterior region of the embryo shows *Pt-fkh* expression in the prospective ectoderm (pr).

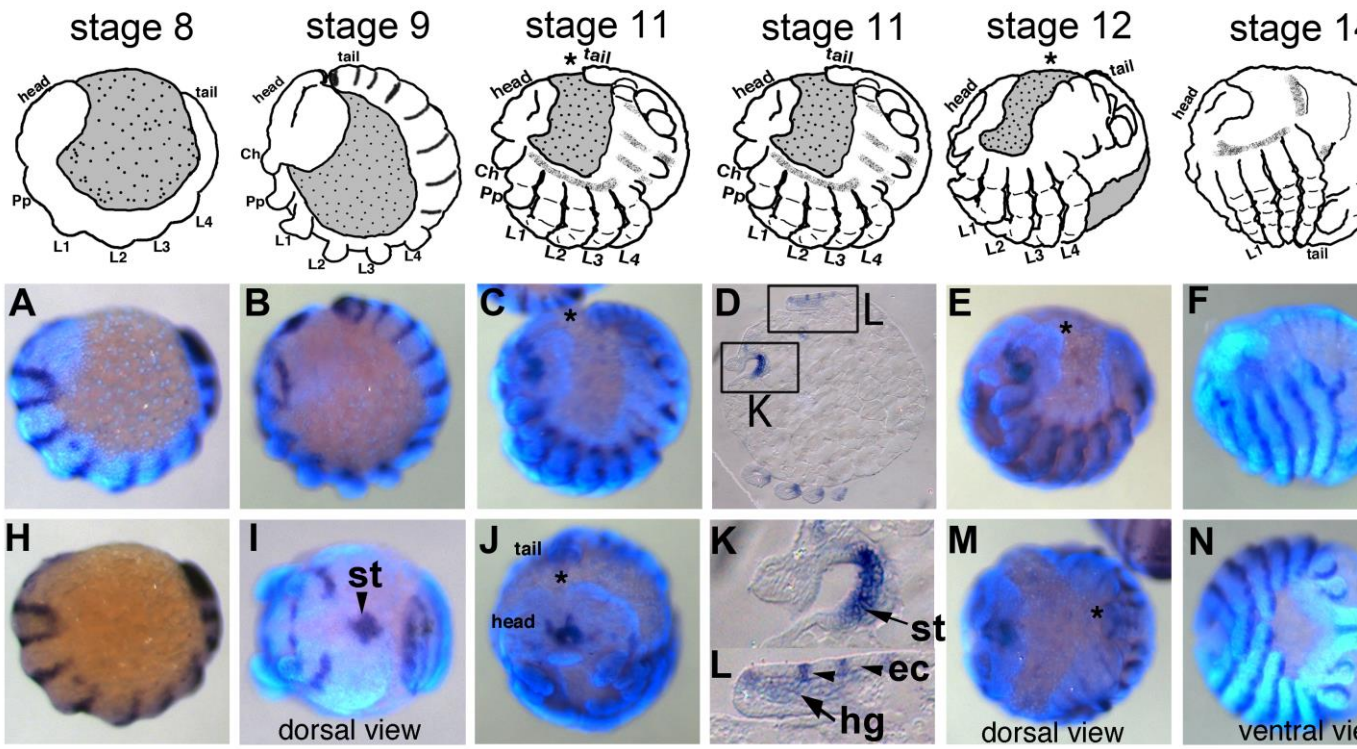

Figure 3: *Pt-hegdehog (Pt-hh)* is expressed in the stomodeum and proctodeum. The top row of panels displays schematic drawings of the images of the embryos below. Anterior is to the left and lateral views are shown in all panels unless specified in the panel. DAPI counterstaining (light blue) highlight the embryo in contrast to the yolk, with the exception of H, and cross sections. D, G, K, L, O, P correspond to longitudinal cross sections. (A,B,C,H,I,J) At stages 8, 9, 11 *Pt-hh* is expressed in each segment, the SAZ and at the stomodeum (st). (D) Longitudinal cross section of an embryo at stage 11 shows that *Pt-hh* is expressed in the anterior (Box K) and in the posterior region (Box L). (K) Higher magnification of the anterior region. *Pt-hh* is expressed in the posterior part of the stomodeum (st). (L) Higher magnification at the posterior region. *Pt-hh* is expressed as ectodermal stripes (ec-arrowheads) and internally in the prospective hindgut (hg-arrow). (E,M) At stage 12, *Pt-hh* expression in the stomodeum and proctodeum are retained, in addition to the segmental pattern. Asterisk marks the dorsal region (E) and posterior region (M). (F,N) At stage 14 *At-hh* expression is observed in the posterior part of the appendages and in the posterior region. (G) Longitudinal cross sections at stage 14 show *Pt-hh* expression in the posterior part of the invaginating stomodeum (Box O) and the inner cells of posterior region (Box P). (O) Higher magnification of the anterior region shows expression in the brain (br) and in posterior region of the stomodeum (st). (P) Higher magnification of the posterior embryonic region shows that the segmental expression in the ectoderm (ec) has disappeared, but it remains in the mesenchymal cells of the hindgut (hg).

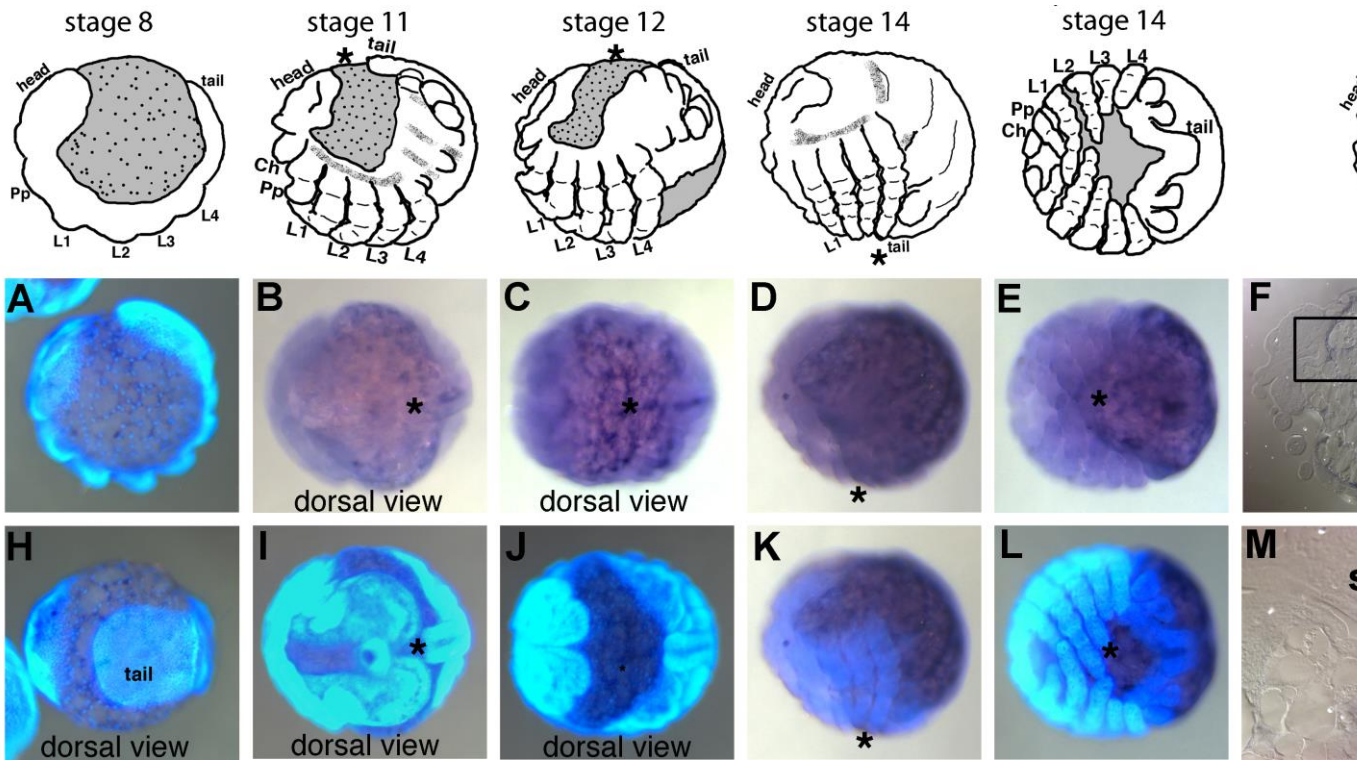

Figure 4: *Pt-hepatocyte-nuclear factor-4 (Pt-hnf4)* is expressed in the extra-embryonic cells and the prospective midgut The top row of panels displays schematic drawings of the images of the embryos below. Anterior is to the left and lateral views are shown in all panels unless specified in the panel. DAPI counterstaining (light blue) highlight the embryo in contrast to the yolk, with the exception of B, C, D, E and cross sections. (A,H) At stage 8 *Pt-hnf4* is expressed in the extraembryonic cells. (B,I) At stage 11 *Pt-hnf4* is expressed in the extraembryonic cells and inside the embryo in the prospective posterior midgut. Asterisks demarcate the dorsal region in order to facilitate the understanding of embryonic orientation. (C,J) At stage 12, when the cells of the posterior region start to migrate *Pt-hnf4* is expressed at the extra-embryonic region. (D,K) At stage 14 *Pt-hnf4* is mainly detected inside the embryo and in a ventral view (E,L) it is possible to observe the ventral closure and the tail (asterisk). (F) Longitudinal cross section at stage 14 shows *Pt-hnf-4* expression along the whole endodermal tube (midgut). (M) Higher magnification of the anterior embryonic region highlights the contact between the ectodermal part of stomodeum (st) - free of *Pt-hnf4*; and endodermal cells from the anterior midgut (am) expressing *Pt-hnf-4.* (G,N) Longitudinal cross section from the posterior embryonic region shows *Pt-hnf4* expression in the endodermal midgut (mg) and denotes the staining (N) in the internal mesenchymal cells at the posterior midgut (pm), but not in the posterior ectodermal cells (pc). This expression pattern is similar to *Pt-hh* at the same developmental stage.

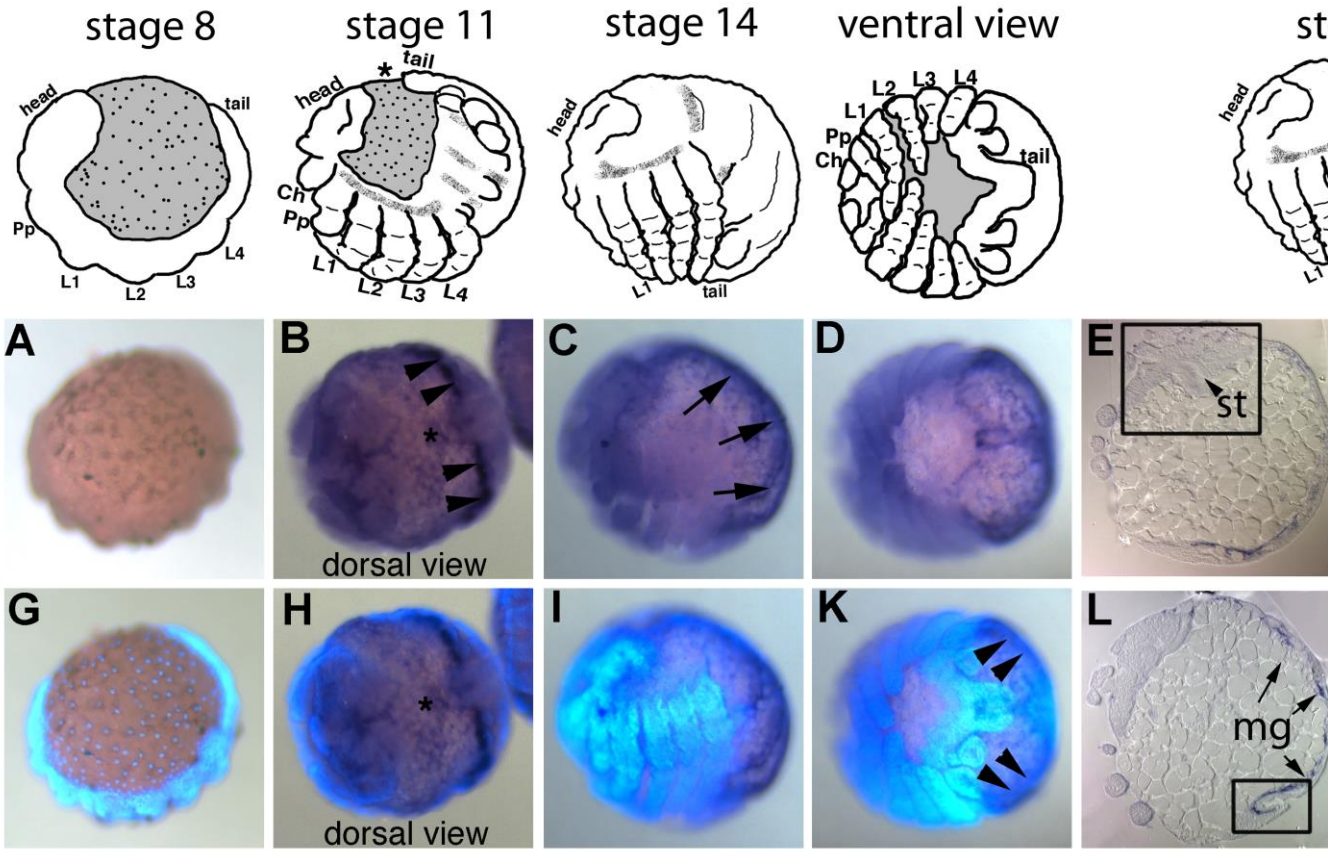

Figure 5: *Pt-serpent (Pt-srp)* is expressed in the extra-embryonic cells and the prospective midgut. The top row of panels displays schematic drawings of the images of the embryos below. Anterior is to the left and lateral views are shown in all panels unless specified in the panel. (A-D) DAPI counterstaining (light blue) highlight the embryo in contrast to the yolk. (A,G) At stage 8 *Pt-srp* expression starts at presumptive extra-embryonic cells. (B,H) At stage 11, cells expressing *Pt-srp* are concentrated in the extraembryonic cells lining the embryo (arrowheads).Asterisk marks the dorsal side of the embryo. (C,I) At stage 14 *Pt-srp* expression appears in the opisthosoma (arrows) and in the extraembryonic region.(D,K) In a ventral view, at stage 14, *Pt-srp* expression is restricted to the inner part of the embryo. (E,F,L,M) Longitudinal cross sections at stage 14 show *Pt-srp* expression in the midgut (mg). (L,M) Unlike *Pt-hnf4, Pt-srp* does not extend towards the posterior internal cell mass and is expressed underlying the dorsal ectoderm. (M) In addition, *Pt-srp* is not expressed at the junction of stomodeum (st)-anterior midgut as observed for *Pt-hnf4*.

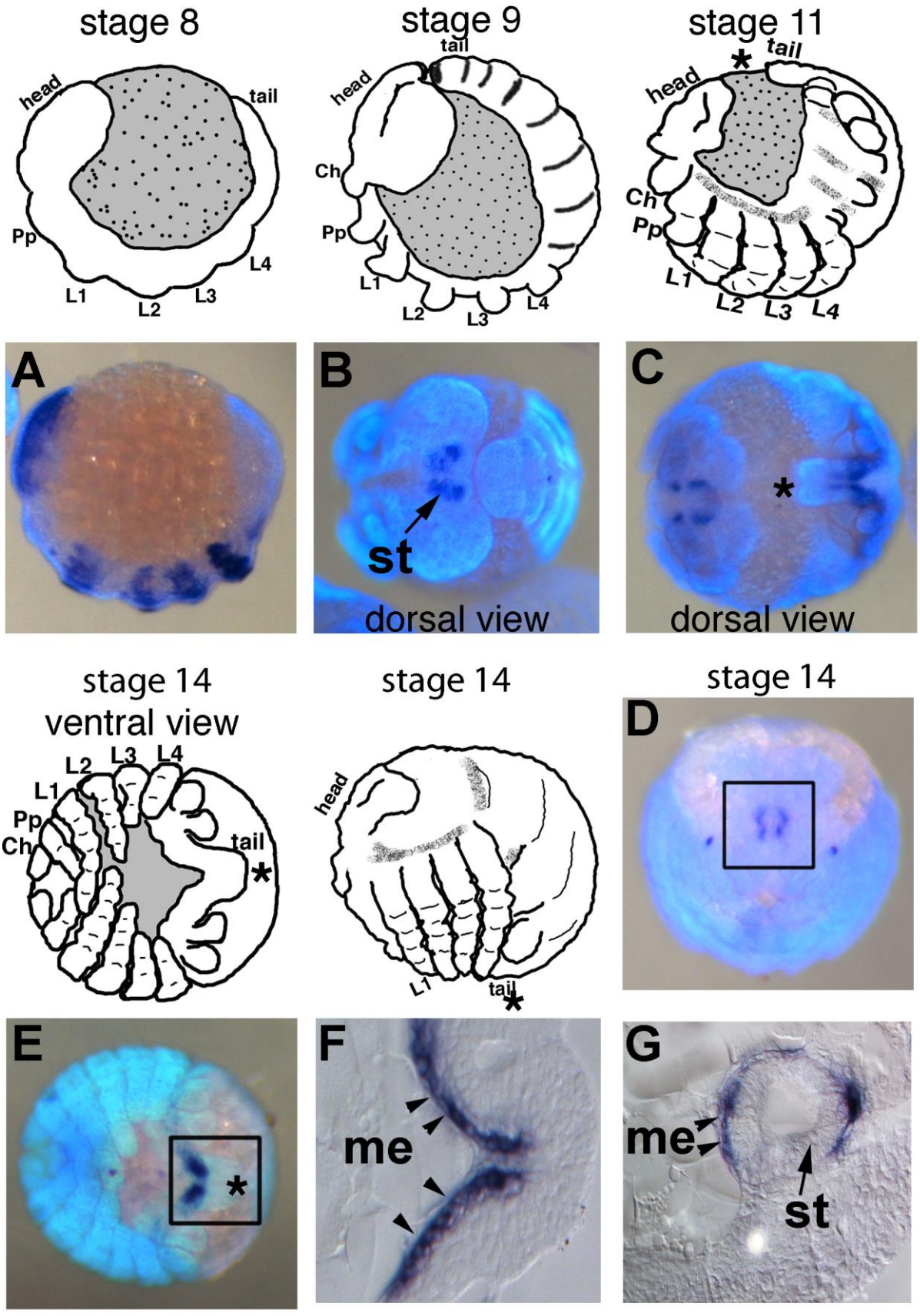
stage 8
head
tail
Pp
L4
L1
L2
L3
stage 9
tail
head
Ch
Pp
L1
L2
L3
L4
stage 11
tail
head
Ch
Pp
L1
L2
L3
L4
A
B
st
dorsal view
C
dorsal view
stage 14
ventral view
L2
L3
L4
L1
Pp
Ch
tail
stage 14
head
L1
tail
stage 14
D
E
F
me
G
me
st

Figure 6: *Pt-twist(Pt-twi)* is expressed transiently in the segmental mesoderm, as well as surrounding the stomodeum and the mesoderm of the SAZ. The top row of panels displays schematic drawings of the images of the embryos below. Anterior is to the left and lateral views are shown in all panels unless specified in the panel. DAPI counterstaining (light blue) highlight the embryo in contrast to the yolk apart from F and G. (A) *Pt- twi* is expressed in the mesoderm of all segments with the exception of the pedipalp. (B) At stage 9, *Pt-twi* is expressed at the head surrounding the stomodeum (st). (C) At stage 11 *Pt-twi* is also detected at the posterior region close to the proctodeum and stomodeum. (D,E) At stage 14, *Pt-twi* expression is maintained in the region flanking the stomodeum in a frontal view (D) and flanking the proctodeum (E, asterisk marks the proctodeum). (F) Transversal cross section at the posterior end shows the two domains of *Pt-twi* expression in the mesenchymal (me) cells at the proctodeum. (G) Transversal cross section at the level of the stomodeum (st) at the anterior region shows *Pt-twi* expression in the mesenchymal cells associated with the gut (me-arrowheads).

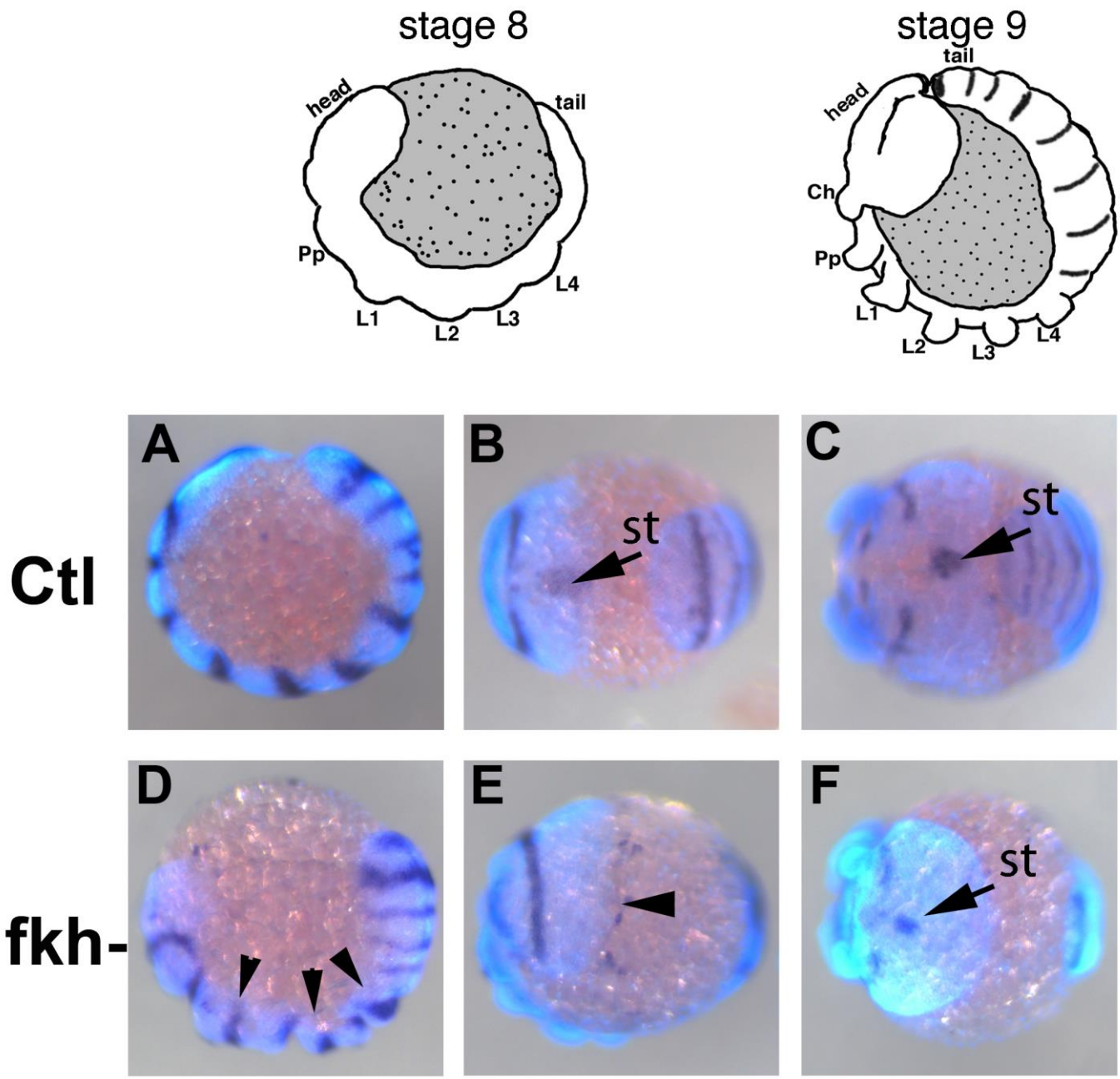

Figure 7: *Pt-fokhead (Pt-fkh)* RNAi embryos display normal segmentation but defects in stomodeum formation. The top row of panels displays schematic drawings of the images of the embryos below. Anterior is to the left and lateral views are shown in all panels unless specified in the panel. DAPI counterstaining (light blue) highlight the embryo in contrast to the yolk. (A,D) Lateral views, head to the left. (B,C,E,F) Top views, with the head to the left. (A,B,C) Control (D,E,F) *Pt-fkh* RNAi. (A,B, D,E) Stage 8 (C,F) Stage 9. (A,D) Control and *fkh* RNAi embryos display eleven *Pt-hh* stripes, but in the control the stripes are straight and in RNAi embryos the stripes are wavy, illustrating the irregular shape of the embryo (arrowheads). (B) Control embryo at stage 8, the stomodeum (st) is highlighted. (E) In strongly affected *Pt-fkh* RNAi embryos, the stomodeum is not observed, but cells expressing *Pt-hh* can be detected at the periphery of the head (arrowhead). (C,F) At stage 9, control embryo (C) and *Pt-fkh* RNAi embryos with a milder phenotype (F) reach stage 9 with normal limb buds, but with a smaller stomodeum (arrow).

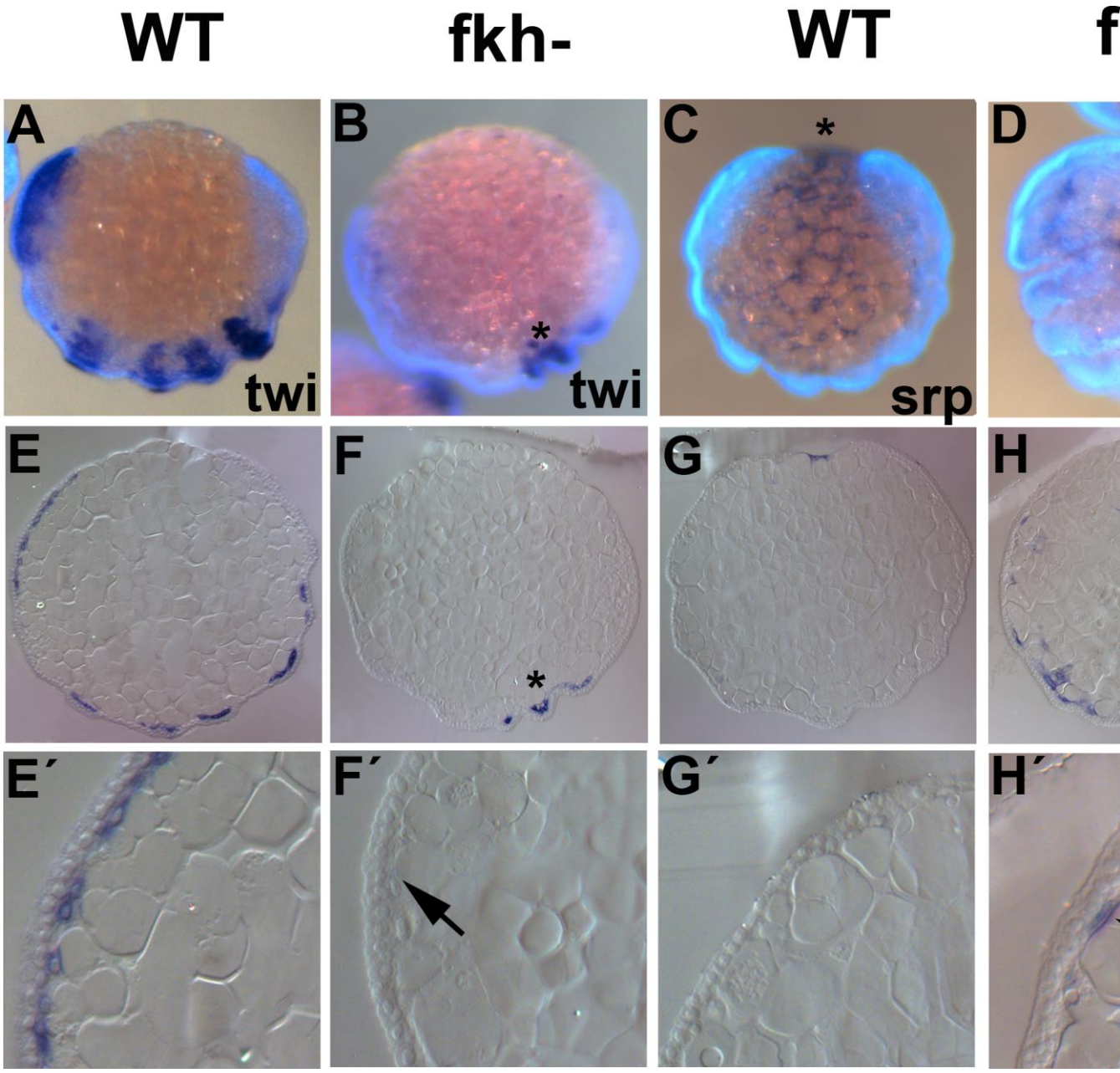

Figure 8: Changes in the expression of *Pt-twi* (mesoderm) and *Pt-srp* (endoderm) after *Pt-fkh* RNAi. Stage 8 of embryonic development. Anterior is to the left and lateral views in all panels. (A,C) Control (B,D) *Pt-fkh* RNAi. (A,E,E´) Control embryos *Pt-twi* whole-mount *in situ* hybridization and respective sagittal cross sections in E,E´. *Pt-twi* is expressed in the mesoderm (me-arrowhead), below the ectoderm. (B,F,F´) In *Pt-fkh* RNAi embryos most *Pt-twi* expression has disappeared, only a small patch of *Pt-twi* expressing cells is still detected at the posterior region of the embryo (asterisk-F). (F,F´) Sagittal cross sections show a few cells below the ectoderm at the anterior region, which do not express *Pt-twi* (arrow). (C,D,G,G´,H,H´) *Pt-srp* expression in control (C,G,G´) and *Pt-fkh* RNAi embryos (D,H,H´).(C) *Pt-srp* is expressed in the extraembryonic cells. Asterisk marks the dorsal region of the embryo. (G) In the sagittal cross section only the dorsal region, which contain extraembryonic cells , expresses *Pt-srp.* (G´) A higher magnification of the anterior region shows that *Pt-srp* is not expressed in these cells. (D,H,H´) After *Pt-fkh* RNAi, extraembryonic cells expressing *Pt-srp* appear disorganized with a patchy distribution (arrow). (H) Sagittal cross section shows ectopic *Pt-srp* expressing cells

beneath the germband cells of the embryo. (H´) A higher magnification of the anterior region of *Pt-fkh* RNAi embryo shows ectopic *Pt-srp* expression below the ectoderm (arrowhead).

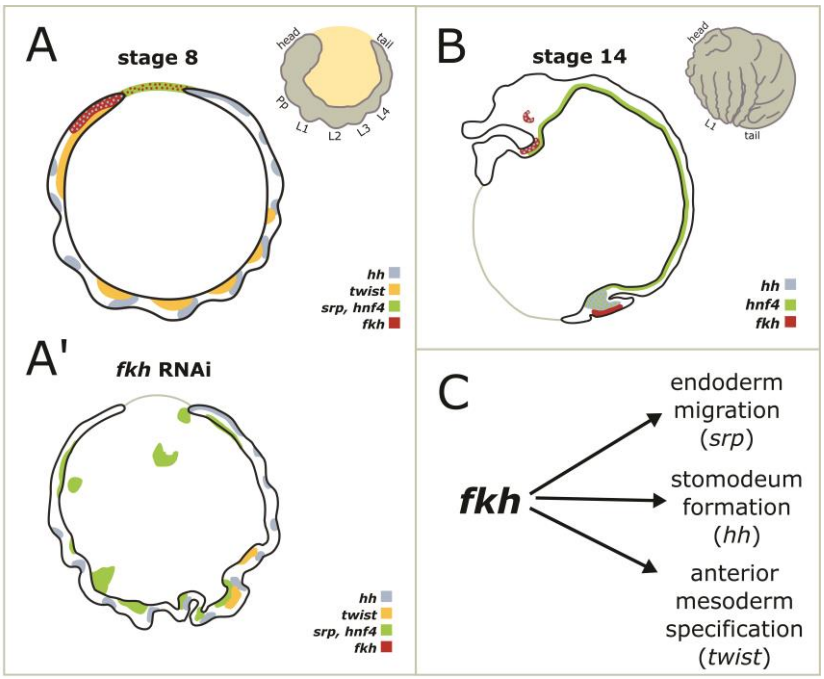

Figure 9: Model of the molecular control of gut formation in the spider *Parasteatoda tepidariorum*. (A,B) Schematic drawings of wild-type sagittal cross sections at stage 8 and stage 14. Major genes and their spatial domains are highlighted with their respective colors. (A) *Pt-hh* (blue) is expressed in a segmental pattern at the posterior region of each segment and the stomodeum, *Pt-twi* (yellow) marks the mesodermal cells underneath the ectoderm, *Pt-srp* and *Pt-hnf4* (green) are expressed in the extraembryonic cells. *Pt-fkh* expression (red) in the ventral midline at stage 8 is not shown in the drawing, since it is presumably not involved in gut patterning. (B) At stage 14 the midgut is established as well as the foregut and hindgut. *Pt-twi* expression was omitted because it is not possible to observe in sagittal sections at this stage. (A') *Pt-fkh* RNAi embryos display several deviations from the wild-type pattern, *Pt-srp* expression (green) indicates incorrect "extraembryonic" cell migration and *Pt-twi* expression (yellow) is reduced to the posterior embryonic region. (C) *Pt-fkh* is required for the activation of the anterior *Pt-twist* expression domain in the mesoderm (Figure 8), in the establishment of the stomodeum, as shown by the analysis of *hh* expression (Figure 7), and for the correct migration of *Pt-srp* expressing cells (Figure 8).

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