Characterisation of the gene regulatory network for posterior segmentation in parasteatoda tepidariorum

Anna Schönauer (2016)

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in Parasteatoda tepidariorum

Anna Schönauer

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always there to convince me that I could do it and that there wasn’t anything else I would rather do.

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Danke Mama, Papa, Judith und Baby Nora für eure Unterstützung in jeglicher Form, Zuspruch und die schöne Zeit zuhause im Schreib-Exil!
Abstract

My PhD project focused on the identification of components and the architecture of the gene regulatory network that controls the formation of the segment addition zone (SAZ) and posterior segments in the spider Parasteatoda tepidariorum. Analysis of the formation and function of the SAZ among arthropods suggests that Wnt and Delta-Notch signaling regulated this process ancestrally in an analogous mechanism to that regulating somitogenesis in vertebrates. However, it remained unknown how the two major signaling pathways interact during the formation of the SAZ and regulate other putatively downstream segmentation genes, such as even-skipped (Pt-eve) and runt (Pt-run-1). Therefore, I studied the interactions between Delta (Pt-Dl) and its receptor Notch (Pt-N) and the Wnt ligand gene Wnt8 (Pt-Wnt8). I showed that Pt-Dl initially activates Pt-Wnt8 in the posterior SAZ, but conversely inhibits Pt-Wnt8 expression in the anterior SAZ. Furthermore, I observed the dynamic expression of Pt-eve and Pt-run-1 in the SAZ and the forming segments, suggesting an important role in posterior development. Moreover my results show that the expression of Pt-eve and Pt-run-1 is regulated by the read out of Delta-Notch and Wnt signaling via caudal (Pt-cad), which might be a mechanism ancestral to all arthropods.

To investigate the function of Wnt signaling in more detail in spiders, I also studied the evolution and expression of Frizzled receptors (Fz) during spider embryogenesis. Four Fz genes (Pt-fz1, Pt-fz2, Pt-fz4a, Pt-fz4b) have been identified in Parasteatoda and analysis of the expression of the frizzled receptor genes throughout embryonic development suggests an involvement in neuroectoderm development, segmentation and development of anterior
structures. Moreover, the early ubiquitous and later segmental expression of *Pt-fz1* shows that this gene is a good candidate receptor for Wnt8 in *Parasteatoda*. 
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Abbreviations

aa amino acid
bp base pairs
°C degrees Celsius
Cas9 caspase 9 endonuclease
C-terminal carboxy-terminal
cDNA copy DNA
Ch cheliceres
CRISPR clustered regularly interspaced palindromic repeats
d dorsal area
DAPI 4-6-diamidino-2-phenylindol
ddH₂O double-distilled water
DIG Digoxigenin
DNA deoxyribonucleic acid
dsRNA double stranded deoxyribonucleic acid
eRNAi embryonic RNAi
h hour
HR homologous recombination
INT/BCIP 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)/ 5-bromo-4-chloro-3-indolyl-phosphate
L walking leg
Lb labrum
ml millilitre
NBT/BCIP nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
NHEJ non-homologous end joining
nt nucleotide
N-terminal amino-terminal
O opisthosomal
PcL precheliceral lobe
PCR polymerase chain reaction
Pp pedipalp
pRNAi parental RNAi
<table>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>RhodB</td>
<td>Rhodamine B isothiocyanate–Dextran</td>
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<tr>
<td>rpm</td>
<td>rotations per minute</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<td>SAZ</td>
<td>segment addition zone</td>
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<td>sgRNA</td>
<td>short guide RNA</td>
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<td>st</td>
<td>stage of development</td>
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<td>Sto</td>
<td>stomodaeum</td>
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<td>wt</td>
<td>wild-type</td>
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1. Introduction

1.1. Evolution & Development

The development of a single-celled zygote into a multicellular organism requires complex molecular mechanisms and tightly regulated developmental programs. Evolutionary developmental biology seeks to compare the genetic regulation of developmental processes in a phylogenetic framework to uncover ancestral and derived features of development and the underlying molecular mechanisms (Carroll, 2008; Gilbert et al., 1996; Hall, 2003). One question that has been of great interest to evolutionary developmental biologists since the emergence of this field is the origin and evolution of segmentation among animals (Davis and Patel, 1999; Davis and Patel, 2002; De Robertis, 2008; McGregor et al., 2009; Tautz, 2004).

1.2. Segments

Various groups of animals exhibit some kind of reiterated body structures (Couso, 2009): echinoderms, hemichordates and molluscs are composed of an array of coelomic cavities of mesodermal origin, separated by epithelia, which has been described as primary segmentation (Tautz, 2004). The formation of segments from undifferentiated posterior tissue, as found in arthropods, annelids and chordates, has been specified as secondary segmentation (Tautz, 2004) (see fig. 1).
Although, these three extant bilaterian phyla are segmented along their antero-posterior axis, it is highly disputed if this characteristic derived from a common segmented ancestor or if the process of segment formation has evolved multiple times independently in the different lineages (Aulehla and Herrmann, 2004; Balavoine and Adoutte, 2003; Chipman, 2010; Couso, 2009; Damen, 2007; Davis and Patel, 1999; Erwin and Davidson, 2002; Graham et al., 2014; Patel, 2003; Peel, 2008; Peel et al., 2005; Pourquie, 2003; Scholtz, 2002; Tautz, 2004). Indeed, vertebrates, arthropods and annelids with segmented bodies are more closely related to unsegmented groups in their phyla than they are to each other (Aguinaldo et al., 1997; de Rosa et al., 1999) (see fig. 1).
1.3. Evolutionary scenarios of segmentation

There are three possible explanations for the evolution of segmentation (Davis and Patel, 1999) (see fig. 2). Firstly, it has been suggested that the common bilaterian ancestor was unsegmented and segmentation has evolved independently in all three bilaterian phyla. In support of this, it has been argued that only minor similarities in the mechanisms for segment formation can be detected, due to the independent evolution of segments (see fig. 2 A) (Davis and Patel, 1999). And furthermore that similarities in regulation have evolved through the parallel recruitment of pre-existing gene-regulatory modules (Chipman, 2010).

The second theory is that the common ancestor of bilateria, the urbilateria, exhibited a segmented body and therefore segmentation is homologous among bilaterian animals. This theory suggests that the whole genetic toolkit for segment formation was present in the common segmented ancestor and this explains similarities in the regulation of segmentation in extant phyla (Davis and Patel, 1999; De Robertis and Sasai, 1996; Kimmel, 1996; Patel, 2003) (see fig. 2 B).

A third hypothesis, is that segmentation evolved independently after the protostome/deuterostome split and therefore segmentation is homologous among arthropods and annelids, but evolved independently in vertebrate chordates (see fig. 2 C). This assumes that the annelid/arthropod clade share a segmented ancestor and hence exhibits a significantly high degree of similarities and major differences compared to vertebrates (Scholtz, 2002). Furthermore, this theory comprises the loss of segmentation in the
unsegmented phyla of Ecdysozoa and Lophotrochozoa (Davis and Patel, 1999).

**Figure 2 | Evolutionary scenarios of segmentation.** (A) Segmentation arose independently in all three phyla. (B) A common segmented ancestor for all three groups with loss of segmentation among the unsegmented phyla. (C) Homology of segments among annelids and arthropods and independent segmentation in chordates. Deuterostomia in blue; Protostomia in green. Asterisks indicated the acquisition of segmentation; solid grey blocks indicate the loss of segmentation (modified after Davis and Patel 1999).

The “segmented common ancestor theory” (see fig. 2 A) was countered with the argument of parsimony: it appears easier to evolve segmentation 3 times independently, than to achieve the loss in numerous unsegmented phyla (Chipman, 2010). In addition it seems unlikely that a complex and highly advantageous trait like segmentation would have been lost (Chipman et al., 2004). Furthermore is has been claimed, that the existence of a segmented bilaterian ancestor is unlikely simply due to a lack of fossil evidence (Erwin and Davidson, 2002). Nevertheless, it has been argued that the losses of segmentation are not impossible to achieve, because the unsegmented groups are clustered and segmentation could have been lost early in the evolutionary history of extant unsegmented phyla (Davis and Patel, 1999).
Indeed the involvement of Delta/Notch signaling in arthropod segmentation, as shown in *Periplaneta* and the spiders *Cupiennius* and *Parasteatoda* as well as in vertebrates has also been interpreted as further evidence for the possibility of a common segmented ancestor (De Robertis, 2008; Oda et al., 2007; Peel and Akam, 2003; Pueyo et al., 2008; Stollewerk et al., 2003). Further support for a common segmented ancestor from analysis of regulatory mechanisms underlying segmentation includes the dynamic expression of *her1*, the vertebrate ortholog of the arthropod pair-rule gene *hairy*, commencing very early on in vertebrate development (Kimmel, 1996). Moreover, it appears that *hedgehog (hh)* is necessary for the maintenance of segmental borders in arthropods and annelids implying a segmented common ancestor of these phyla (Dray et al., 2010; Farzana and Brown, 2008; Ingham and McMahon, 2001). It has also been suggested that a gradient emerging from the anterior, like the *Drosophila* bicoid gradient, would not be able to pattern the posterior in short germ arthropods as it cannot reach the posterior, hence a posterior signaling centre must have regulated segmentation ancestrally. Hence, the homeodomain transcription factor *caudal (cad)*, which is involved in patterning the posterior of *Drosophila* embryos, as well as in other arthropods like its Cdx orthologs in vertebrates, again evidences similarities in posterior development between distantly related segmented phyla (Lall and Patel, 2001).

On the contrary, other authors have claimed that some parts of the genetic toolkit, like signaling pathways and their individual components, like transcription factors are employed in many different aspects of development
and hence, to derive homology and a common origin from such genetic modules appears incorrect (Erwin and Davidson, 2002). Another explanation for the observation of common mechanisms, with a varying composition of factors involved and changes in their regulation, is the generation of the GRN of segmentation by convergent evolution. In this process, an already established network, acquires new components after an evolutionary event, like a whole genome duplication, through co-option of the duplicated factors (Minelli, 2015). It has been suggested that for example the generation of repeated structures by the Delta-Notch and the Wnt signaling pathways displays a co-opted function of their ancient role in axis elongation (Chipman, 2010).

1.4. Mechanisms of Segmentation

1.4.1. Segmentation in Vertebrates

Three different developmental events, the formation of somites, the subdivision of the hindbrain into rhombomeres and the formation of the pharyngeal arches are regarded as segmentation processes in vertebrates (Graham et al., 2014). However, only the sequential formation of the somites from the pool of undifferentiated presomitic mesoderm (PMS) cells, displays an analogous mechanism to the segmentation process in arthropods (Graham et al., 2014). It is important to emphasise, that vertebrate somites arise from the mesoderm, in contrast to ectodermal arthropod segments. However, the mode of segment or somite formation, respectively from a posterior pool of
undifferentiated cells, regulated by dynamic gene expression is at least an analogous mechanism.

Somite formation in vertebrates is regulated by opposing gradients of gene expression, which sequentially subdivide the PMS into smaller subunits (Kageyama et al., 2012; Pourquie, 2001) (Fig. 3). In the ‘clock and wavefront’ model for somite formation first proposed by Cooke and Zeeman, 1976 the periodic production of somites in the PSM is regulated through the cyclical expression of members of the hairy/enhancer of split (Hes)-gene family (Takke and Campos-Ortega, 1999) (see fig. 3). In the mouse, Hes7 forms an auto-regulatory feedback loop, in which the unstable Hes7 protein represses its own transcription resulting in oscillating Hes7 expression in the PSM (Nomura-Kitabayashi et al., 2002). However, the synchronicity of expression in the PSM is only achieved through cell-to-cell signaling via Delta/Notch, where Delta activates Notch in a neighboring cell to initiate Hes7 expression (Cooke, 1998; Jiang et al., 2000). The interface between the site of somite differentiation and the posterior oscillatory gene expression has been described as the wave front (Kageyama et al., 2012). The wave front is generated by a rostral to caudal FGF gradient (Dubrulle et al., 2001) (see fig. 3). The low levels of FGF and high levels of Notch in the anterior finally trigger the expression of a regulator of somite formation, Mesp2 (Kageyama et al., 2012; Saga, 2007) (see fig. 3). Additionally, a gradient of Wnt3a helps to set the boundary for somite formation and maintains oscillations (Aulehla and Herrmann, 2004). Low levels of Wnt3a, inhibit the oscillation and allow differentiation to occur (Aulehla and Herrmann, 2004) (see fig. 3)
Introduction

1.4.2. Segmentation in Annelids

Segment addition in annelids occurs at larval and juvenile stages. In the marine annelid, *Platynereis dumerilii* (*Pdu*), the first signs of morphological segmentation become apparent at the swimming larval stage (Fischer et al., 2010). After an elongation phase, where the larva develops into a worm with three distinct segments, the larva settles and continues to form segments sequentially from the segment addition zone (SAZ) (de Rosa et al., 2005; Fischer et al., 2010).

*Figure 3 | Vertebrate somitogenesis.* Delta-Notch signaling at the posterior conveys the periodic signal in the presomitic mesoderm (PSM, light blue), where it activates Hes-gene expression. Hes-genes form an auto-regulatory feedback loop, which leads to oscillatory expression. Wnt and FGF signaling establish opposing gradients in the PSM. *Mesp* expression at the wavefront is activated and allows cell differentiation and hence somite formation in periodic intervals.

1.4.2. Segmentation in Annelids

Segment addition in annelids occurs at larval and juvenile stages. In the marine annelid, *Platynereis dumerilii* (*Pdu*), the first signs of morphological segmentation become apparent at the swimming larval stage (Fischer et al., 2010). After an elongation phase, where the larva develops into a worm with three distinct segments, the larva settles and continues to form segments sequentially from the segment addition zone (SAZ) (de Rosa et al., 2005; Fischer et al., 2010).
The relative expression domains of the segment polarity gene orthologs *hedgehog* (*Pdu-hh*), *engrailed* (*Pdu-en*) and the Wnt ligand *Pdu-Wnt1/wg* (Dray et al., 2010; Prud'homme et al., 2003) are reminiscent of the parasegmental boundary described in *Drosophila*, where *wg* is expressed anterior of *en* overlapping with *hh* expression (Martinez-Arias and Lawrence, 1985) (see also section 1.3.3.1. “The *Drosophila* paradigm”).

Furthermore, inhibition of Hedgehog (Hh) signaling in *Platynereis* leads to the loss of the segmental groove, the segments acquire an ovoid shape and germ band elongation is disrupted (Dray et al., 2010). Hence, Hh signaling is critical for boundary maintenance in nascent segments prior to morphological segmentation in this annelid (Dray et al., 2010), as has been found in arthropods (Farzana and Brown, 2008).

Furthermore, expression data of the *Platynereis* homologues of the two segmentation genes *caudal* (*Pdu-cad*) and *even-skipped* (*Pdu-eve*) in the SAZ and in forming segments of *Platynereis*, suggest that they have a function in segmentation because they are expressed in domains and at developmental time points comparable to what has been observed in arthropods (de Rosa et al., 2005). Phylogenetic analysis identified 13 *Hes/Hey*-related genes in *Platynereis* (Gazave et al., 2014), which are known to be part of the oscillatory gene expression and downstream targets of Delta/Notch signaling in vertebrate somitogenesis (Kageyama et al., 2012). However, there is no functional data available for the *Hes/Hey*-related genes in *Platynereis*.

Leeches undergo direct embryonic development and form segments sequentially from teloblast cells, embryonic stem cells, which in turn form
columns of segmental founder cells (Weisblat et al., 1984). Morphologically, the first signs of segmentation become obvious after the fusion of the two bilateral germ bands, when the germ band divides from anterior to posterior into repeated units (Bissen and Weisblat, 1989; Zackson, 1982). However, expression data of the even-skipped homologs in the leeches Helobdella robusta (Hro) and Theromyzon trizonare (Ttr) do not support a role for these genes in segmentation (Song et al., 2002), unlike in Platynereis (de Rosa et al., 2005). Indeed knockdown of Hro-eve suggests a role in cell proliferation and neurogenesis, rather than segmentation in this leech (Song et al., 2002).

Furthermore, analysis of hes-genes in the two leeches (Hro-hes, Ttr-hes) does not support a function in segmentation, again in contrast to Platynereis (Song et al., 2004).

Whilst the molecular organization of segment boundaries in Platynereis displays similarities with the Drosophila parasegmental boundaries and the expression of the segmentation genes caudal and even-skipped suggest a role in segmentation, studies in the two leeches could not confirm an involvement of even-skipped in segmentation (de Rosa et al., 2005; Dray et al., 2010; Martinez-Arias and Lawrence, 1985; Song et al., 2002).

Although the analysis of the relative expression patterns of segmentation gene orthologs show analogy with vertebrates and arthropods (Dray et al., 2010; Prud'homme et al., 2003), the diverse developmental processes and the limitations of functional tools in annelids make the study of segmentation and also the comparison with other segmented phyla challenging (Balavoine, 2014).
1.4.3. Segmentation in Arthropods

The phylum name Arthropoda is derived from the Greek words for “jointed” (arthros) and “feet” (podes), as all arthropods exhibit jointed appendages. All arthropods also display a sclerotized cuticle, the exoskeleton, which encloses the whole body and is shed during growth. Furthermore, the arthropod body is composed of segments along the antero-posterior axis and their nervous system is located ventrally (Anderson, 1973; Scholtz, 1998). It has been hypothesised that the modular body plan of arthropods has facilitated flexibility for adaption to the requirements of the diverse habitats and so has majorly contributed to the success of these animals (Stansbury and Moczek, 2013).

The Drosophila paradigm

Segmentation in arthropods has been most intensely studied in Drosophila, which employs a derived mode of segment formation among arthropods where the specification of the cephalic, thoracic and abdominal segments occurs almost simultaneously along the anterior-posterior axis (Davis and Patel, 2002; Ingham, 1988; Lawrence, 1992; Nusslein-Volhard and Wieschaus, 1980; Pankratz and Jäckle, 1993; Peel et al., 2005). This specification of segments in the fruit fly is regulated by a well characterised segmentation cascade (Scott and Carroll, 1987) (see fig. 4).
Introduction

Figure 4 | The *Drosophila* segmentation gene cascade. Maternal transcripts bicoid (BCD) and nanos (NOS) are localized to the anterior and posterior pole, respectively; hunchback (HB) and caudal (CAD) are expressed ubiquitously (Step 1). The output of the maternal effect genes activates zygotically expressed gap genes (tailless, tll; giant, gt; Krüppel; knirps) at specific positions along the A-P axis (Step 2). The primary (*hairy, even-skipped, runt*) and secondary (*fushi tarazu, paired*) pair-rule genes interpret the aperiodic expression of the maternal effector and gap genes, to generate a periodic stripe pattern, predicting the parasegmental boundaries (Step 3). Odd- and even-numbered segments express different combinations of pair-rule genes. The parasegmental boundary is established between the *engrailed* domain anteriorly and the *wingless* domain posteriorly (Step 4). Picture is taken from (Peel et al., 2005).
Initially, localized maternal transcripts are translated and establish long-range transcription factor gradients in the syncytial blastoderm, which in turn regulate zygotic downstream factors (Rivera-Pomar et al., 1995; St Johnston and Nusslein-Volhard, 1992). Unique to higher diptera, including *Drosophila*, is the maternally deposited factor *bicoid*, which is translated upon fertilisation to form an anterior to posterior gradient that activates gap genes in a concentration dependent manner (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988b; Lehmann and Nusslein-Volhard, 1991; St Johnston and Nusslein-Volhard, 1992; Stauber et al., 1999) (see fig 4, step1). Bicoid has also been shown to directly repress the translation of the initially uniformly distributed maternal factor *caudal (cad)* in the anterior (Macdonald and Struhl, 1986).

Subsequently, maternal coordinate genes trigger the asymmetric expression of gap genes within the blastoderm (see fig. 4, step 2). Gap genes in turn activate pair-rule gene expression and spatially regulate and refine their alternating expression together with maternal inputs and auto-regulatory feedback of pair-rule genes themselves (Carroll, 1990; Frasch and Levine, 1987; Gaul and Jackle, 1990). The double-segmental pair-rule pattern further activates the single-segmental expression of segment polarity genes, delineating the borders of the parasegments and compartments of the future segments (Rivera-Pomar and Jackle, 1996) as the parasegments, are out of phase with the true segmental boundaries (Martinez-Arias and Lawrence, 1985) (see fig. 4, step 4). Typically, * engrailed (en)* is expressed in the anterior portion of the parasegment, which corresponds to the posterior of the future segment and *wingless (wg)* specifies the posterior of the parasegment.
(Kornberg et al., 1985; Martinez-Arias and Lawrence, 1985) (see fig. 4, step 3 and 4 and fig. 5). *En* activates the expression of *hedgehog (hh)*, which binds the *patched (ptch)* receptor on *wg* expressing cells. This in turn activates *wg*, maintaining its expression and thereby defining the parasegmental borders (Heemskerk et al., 1991; Ingham et al., 1991; Mohler and Vani, 1992) (see fig. 5). *en* and *wg* do not only delineate the parasegments in *Drosophila*, but have been shown to exhibit conserved relative expression in other arthropods (Damen, 2007; Patel et al., 1989b).

**Figure 5 | Maintenance of the parasegmental boundary in Drosophila.** After the establishment of the parasegmental boundaries through the pair-rule genes, *wg* protein diffuses to the neighbouring cells, binds to the frizzled (*fz*) receptor on *en* expressing cells (Bhanot et al., 1996). Subsequently *en* activates *hh* expression, which in turn binds the *patched (ptch)* receptor in *wg* expressing cells, leading to an activation of *wg* expression (Ingham et al., 1991; Mohler and Vani, 1992).

Finally, the combinatorial expression of gap, pair-rule and segment polarity genes regulate the Hox genes, which determine the segment identity (Affolter et al., 1990; Akam, 1987; Harding et al., 1985; Irish et al., 1989; Lewis, 1978; Pearson et al., 2005).
In contrast to *Drosophila*, most other arthropods are short or intermediate germ arthropods, which specify a species-specific number of anterior segments at the blastoderm stage and form posterior segments consecutively from the SAZ, and in some cases add segments also during post embryonic stages (Davis and Patel, 2002) (see fig. 6).

This sequential addition of segments also occurs in a cellularised environment, suggesting that different molecular mechanisms may regulate segment formation in long germ arthropods compared to short germ arthropods (Peel et al., 2005). For example in *Drosophila* the patterning of the future segments occurs through long-range transcription factor gradients, which is not possible in a cellular environment (Davis and Patel, 2002; Pankratz and Jäckle, 1993). However, despite the common conception that all future segments of *Drosophila* are specified simultaneously, a succession in the appearance of pair-rule and segment polarity stripes can be observed, with anterior stripes
showing up first (Bothma et al., 2014; Janssens et al., 2014; Pankratz et al., 1990). These observations led to the suggestion that the successive segmentation gene expression appearance is a remnant of secondary growth and the ancestral cell-cell based mode of segmentation was not entirely replaced by a morphogen gradient driven process in *Drosophila* (Tautz, 2004). Despite the in depth knowledge about segmentation in the long germ insect *Drosophila*, the gene regulatory network (GRN) responsible for the set up of the SAZ and the sequential segment formation from this tissue in short germ arthropods remains poorly understood.

**Segmentation in short germ arthropods**

**Orthologs of maternal factors**

In *Drosophila*, Bicoid is an important maternal factor, however it represents a derived characteristic, not found outside of Diptera (Driever and Nusslein-Volhard, 1988a; McGregor, 2005; Stauber et al., 1999). Cad is also important during Drosophila embryogenesis and is distributed in a reciprocal gradient to Bcd along the anterior-posterior axis with the maximum at the posterior (Mlodzik and Gehring, 1987).

Moreover, *cad* is expressed in the embryonic posterior of many arthropods and *cad* RNAi knockdown experiments cause posterior defects (Copf et al., 2004; Dearden and Akam, 2001; Olesnicky et al., 2006; Shinmyo et al., 2005). Furthermore, *cad* regulates the expression of anterior gap genes in *Tribolium* and *Gryllus*, which suggests a role at the top of the segmentation gene cascade (Copf et al., 2004; Shinmyo et al., 2005).
The most recent studies in *Tribolium* revealed that the graded expression of *caudal* (*Tc-cad*) also modulates the frequency of *even-skipped* (*Tc-eve*), resulting in oscillating waves of *Tc-eve* expression (Copf et al., 2004; El-Sherif et al., 2014). Hence, it has been suggested that *Tc-cad* acts a morphogen, which regulates the rate of pair-rule gene expression in *Tribolium* (El-Sherif et al., 2014). These findings are in great contrast to the static *even-skipped* expression, regulated by the combinatorial action of gap genes in *Drosophila* (Frasch and Levine, 1987).

**Gap gene orthologs**

In most short germ arthropods, gap gene expression commences early in the germ rudiment in broad domains overlapping several future segments, however the relative expression is not conserved (Bucher and Klingler, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Liu and Patel, 2010). Furthermore, the functional analysis of gap genes in e.g. *Tribolium*, *Oncopeltus* and *Gryllus* shows a more complex picture than in *Drosophila* and in some cases the knockdown causes malformation rather than a lack of several adjacent segments (Bucher and Klingler, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Mito et al., 2005; Schroder, 2003).

In *Parasteatoda*, the development of the prosoma requires *hunchback* (*hb*) and *distal-less* (*Dll*) expression and there is evidence that these genes perform gap gene-like functions in this spider (Pechmann et al., 2009; Schwager et al., 2009). *Dll* is expressed in a broad domain in the presumptive L1 segment at early stages, whereas the homologous mandibular segment in
insects lacks \textit{Dil} and is expressed in the homologous segment only at low levels in other mandibulate arthropods (Pechmann et al., 2011).

In insects, \textit{hb} mainly regulates pair-rule and HOX gene expression and causes homeotic transformations in knockdown experiments (Liu and Kaufman, 2004a; Marques-Souza et al., 2008; Mito et al., 2005). In \textit{Parasteatoda} \textit{hb} is expressed before morphological segmentation and causes a loss of adjacent segments in RNAi experiments (Schwager et al., 2009). Therefore, it has been proposed that \textit{hb} acts like a gap gene in the spider and is responsible for the correct expression of target genes, rather than HOX gene regulation (Schwager et al., 2009).

**Pair-rule gene orthologs**

In the short germ insect \textit{Tribolium} pair-rule genes are expressed with double-segmental periodicity like in \textit{Drosophila}, which resolves into single-segmental expression through splitting of those primary stripes or intercalation of secondary stripes (Brown et al., 1997; Goto et al., 1989; Maderspacher et al., 1998; Patel et al., 1994). However, the knockdown of \textit{Tc-eve}, \textit{Tc-run} and \textit{Tc-odd} does not result in the classic pair-rule phenotypes exhibiting the loss of alternating segments, but causes severe truncations in all three cases (Choe et al., 2006). On the other hand, \textit{Tc-slp} and \textit{Tc-prd}, were shown to occur in double segmental periodicity and produce pair-rule gene phenotypes with the loss of alternating segments in RNAi experiments (Choe and Brown, 2007). These observations led to the conclusion, that pair-rule genes act on two different functional levels and the hierarchy as described in \textit{Drosophila}, is maintained in \textit{Tribolium} (Choe et al., 2006).
Whilst primary pair-rule genes are regulated by maternal factors and gap genes in *Drosophila*, a different mechanism of pair-rule regulation has been proposed in *Tribolium* (Choe et al., 2006; Frasch and Levine, 1987): Tc-eve has been shown to activate Tc-run expression, which in turn activates T-odd expression (Choe et al., 2006). The subsequent repression of Tc-eve by Tc-odd finally closes a pair-rule gene circuit in *Tribolium* (Choe et al., 2006). Hence, it can be concluded that certain aspects of the derived *Drosophila* segmentation still represent ancestral aspects of insect segmentation, but there is also variation in the precise regulation and roles of pair-rule genes (Choe and Brown, 2007; Patel et al., 1994).

In contrast to the double segmental periodicity of pair-rule genes in some insects, the orthologues of these genes exhibit single segment periodicity in short germ arthropods like spiders and during the addition of the final few segments in the centipede *Strigamia* (Brena and Akam, 2013; Leite and McGregor, 2016; Schoppmeier and Damen, 2005a).

For example, in the spider *Cupiennius salei*, the primary pair-rule gene orthologues hairy (*Cs-h*), even-skipped (*Cs-eve*) and runt (*Cs-run*) are expressed dynamically in the SAZ and with single-segmental periodicity in nascent segments (Damen et al., 2000). Furthermore, the secondary pair-rule gene orthologs paired (*Cs-opa*), Cs-odd related 1 (*Cs-odd-r1*) and sloppy-paired (*Cs-slp*) are likely to be involved in segmentation, due to their expression in the anterior SAZ and nascent segments (Damen et al., 2005).

The *Strigamia* pair-rule gene ortholog expression of Sm-eve, Sm-run, Sm-odd, Sm-h precede morphological segmentation and establish a double-segmental pattern in the peri-proctodeal area, and were therefore suggested to perform a
homologous function to primary pair-rule genes in *Drosophila* (Chipman and Akam, 2008; Chipman et al., 2004). Initially, *Sm-eve1* is expressed in a double-segmental pattern, out of phase with the double-segmental *Sm-Dl* expression (Brena and Akam, 2013). Subsequently, *Sm-eve1* and *Sm-Dl* were observed to resolve into single segmental stripes through splitting or intercalations (Brena and Akam, 2013). However, a detailed analysis of the expression of *Sm-Dl* and *Sm-eve1* revealed a striking change in pair-rule periodicity in the centipede, towards the end of the segmentation process (Brena and Akam, 2013). Interestingly, for the formation of the approximately 10 last trunk segments the initially dynamic expression slows down and *Sm-eve* is expressed uniformly in the peri-proctodeal area, which resolves into single-segmental stripes, co-expressed with *Sm-Dl* (Brena and Akam, 2013). The dynamic expression in the posterior of the centipede, with the switch from double to single-segmental periodicity, has lead to the conclusion that oscillatory gene expression with single-segmental pair-rule gene expression represents the ancestral mechanism for the generation of segments. Furthermore, it has been suggested that the double-segmental patterning of the majority of trunk segments may be an adaption to the rapid development of centipedes (Brena and Akam, 2013; Damen, 2004; Leite and McGregor, 2016).

**Segment polarity gene orthologs**

Analysis of the segment polarity orthologs in the *Cupiennius* implies that the functional organisation of parasegmental boundaries is an ancestral feature of arthropods (Damen, 2002). In the spider, two copies of *engrailed* have been
identified (Cs-en1, Cs-en2) and wingless (Cs-wg) together with a Wnt ligand (Cs-Wnt5-1) have been proposed to define the parasegmental boundary. The combined expression of Cs-en1 and Cs-en2 cover the posterior of the functional unit, where Cs-wg and Cs-Wnt5-1 in conjunction specify the domain just anterior to that. Indeed, the relative expression of wg and en expression at the segmental borders appears to be conserved across all arthropods (Jaynes and Fujioka, 2004; Marie and Bacon, 2000; Patel et al., 1989a; Patel et al., 1989b).

**Wnt and Delta-Notch signaling in arthropod segmentation**

While Delta-Notch signaling is not involved in regulating *Drosophila* segmentation (Peel et al., 2005), it has been found that this pathway is crucial for segment formation in several short germ arthropods.

In the cockroach *Periplaneta*, it has been demonstrated that components of the Delta-Notch pathway are expressed in dynamic stripes in the SAZ and the nascent segments emerging from the posterior (Pueyo et al., 2008). Furthermore, *Pa-N* is crucial for SAZ establishment and maintenance, segment formation and expression of the downstream factors *Pa-h* and *Pa-en* (Pueyo et al., 2008). Note also that *Pa-h* exhibits single segmental periodicity, unlike in *Drosophila* (Pueyo et al., 2008). Further analysis of the molecular mechanism of segment addition in *Periplaneta*, revealed that the sequential formation of segments is regulated by oscillating levels of gene expression, which originate from the SAZ (Chesebro et al., 2012) (see fig. 7). More specifically, *Pa-Wnt1* initially activates *Pa-cad* in the posterior, forming a signaling centre responsible for the set up and maintenance of the SAZ (see
fig. 7). *Pa-cad* is expressed in a broad domain, maintaining SAZ cells in an undifferentiated state (see fig. 7). Subsequently, *Pa-Wnt1* expression activates *Pa-Delta* in the posterior and establishes a positive feedback loop, which regulates cyclic expression of *Pa-Dl* (see fig. 7). It is thought that if *Pa-Delta* expression reaches a certain threshold it can pass through the *Pa-cad* domain and trigger segmentation gene expression like *Pa-en* anteriorly (Chesebro et al., 2012) (see fig. 7).

![Figure 7 | The Periplaneta posterior organiser.](image)

The involvement of Delta/Notch signaling during segmentation in a chelicerate was first shown in *Cupiennius*, where *Delta-1, Cs-Delta-2* and *Notch (Cs-N)*
are expressed in the SAZ and resolve into stripes in nascent segments later, prior to morphological segmentation (Stollewerk et al., 2003). Functional analysis of all three genes using RNAi knockdown resulted in malformation of the segments with indistinct borders, irregular shapes and an enlarged SAZ. Moreover, the expression pattern of Cs-h was perturbed in the SAZ and the forming segments in these RNAi embryos (Stollewerk et al., 2003). Therefore the authors concluded that Notch-signaling is necessary for segment patterning and the establishment of sharp segmental borders in Cupiennius (Stollewerk et al., 2003).

It was also shown that Delta/Notch signaling in spider segmentation acts via the down stream targets Suppressor of hairless and Presenillin (Damen, 2002; Stollewerk et al., 2003). The two copies of Suppressor of hairless (Cs-Su(H)-1, Cs-Su(H)-2) and Presenillin (Cs-Psn) are expressed ubiquitously at early stages and specific Cs-Su(H)-2 segmental expression comes on at later stages. RNAi knockdown of Cs-Su(H)-1 and Cs-Su(H)-2 caused identical phenotypes, where segmentation is blocked after the formation of three irregular-shaped opisthosomal segments and an enlarged SAZ. Moreover, the expression of both Cs-en and Cs-h is lost in the Cs-Su(H)-1 and Cs-Su(H)-2 knockdown embryos (Damen, 2002; Schoppmeier and Damen, 2005b; Stollewerk et al., 2003). The effect of Cs-Psn knockdown is similar to what has been observed for Cs-Su(H)-1 and -2, however, five segments are formed before posterior development stops. Also, segment shape and size are affected, but in contrast to Cs-Su(H)-1 phenotypes, the head lobes do not develop properly and appendages in the prosoma are shortened or missing, but the SAZ is unaffected. Expression of Cs-h and Cs-Delta-1 is abolished in
the posterior of the Cs-Psn RNAi embryos (Schoppmeier and Damen, 2005b). These results show that Delta-Notch signaling in spiders activates the same downstream cascade as in vertebrate somitogenesis and that Notch signaling in spiders is responsible for SAZ and segment border formation analogous to it’s function in vertebrates (Ferjentsik et al., 2009).

In Parasteatoda, the ligand Delta (Pt-Dl) and the receptor Notch (Pt-N) exhibit expression in the SAZ and stripes in opisthosomal segments (Oda et al., 2007). Functional analysis of these genes also shows that both are required for the development of the SAZ and subsequent generation of segments. Knockdown of Pt-Dl or Pt-N cause an abnormal thickening of the tissue at the developing posterior of the germ disc. These early phenotypes develop into different posterior phenotypes ranging from embryos with reduced opisthosomal tissue, with a normal prosoma, to a complete loss of the opisthosoma and disorganised anterior regions of these germ bands. Cells in the aggregated caudal region of Pt-Dl or Pt-N RNAi embryos strongly express the mesodermal marker twist (Pt-twi) (Yamazaki et al., 2005) and lack expression of the posterior determinant gene caudal (Pt-cad) (see fig. 8). This indicates that the specification of caudal ectoderm fails in Pt-Dl RNAi embryos due to the insufficient downstream activation of Pt-cad and the over expression of Pt-twi (Oda et al., 2007).

The Wnt-signaling pathway is also crucial for posterior spider segments. In Parasteatoda, knockdown of the ligand Wnt8 (Pt-Wnt8) results in malformation and truncation of the opisthosoma. Moreover, it was shown that the lack of Pt-Wnt8 leads to a misregulation of Pt-Dl and the Delta-Notch
downstream factor *hairy* (*Pt-h*) in the SAZ (McGregor et al., 2008b). Like in vertebrates, these results suggest that Wnt signaling is involved in the regulation of Delta/Notch signaling in spiders. It has been speculated that *Pt-Wnt8* might be responsible for establishing and maintaining the pool of posterior, undifferentiated SAZ cells and the specification of caudal ectoderm through repression of *Pt-twi* and activation of *Pt-cad* (McGregor et al., 2009).

Summarising the results in *Parasteatoda*, the loss of *Pt-Delta* or *Pt-Wnt8* causes opisthosomal truncations in the most severe cases (McGregor et al., 2008b; Oda et al., 2007). Furthermore, it has been shown that both signaling pathways regulate the posterior determinant gene *Pt-cad* (McGregor et al., 2008b; Oda et al., 2007) (see fig. 8).

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**Figure 8 | Summary of the GRN of posterior segmentation in *Parasteatoda*.** It has been shown in RNAi knockdown experiments that Delta and Notch activate (orange arrow) caudal and repress twist expression in the posterior of the SAZ (Oda et al., 2007). Further it could be shown that Wnt8 represses twist and hairy, but activates caudal (green arrow) expression in the spider (McGregor et al., 2008b).
Components of the Delta-Notch pathway have also been suggested to be involved in segmentation of the centipede *Strigamia maritima* (Brena and Akam, 2013). Dynamic levels of gene expression have been described in the peri-proctodeal area, which resolve into stripes in the developing trunk segments for *Sm-eve2* and *Sm-Delta*. At the blastoderm stage oscillatory expression of those two genes arises from around the blastopore and later from two lateral patches at the forming proctodeum, suggesting that those structures embody the posterior signaling centre. A change in expression of those genes from oscillatory double segmental to static single segmental expression occurs in order to pattern the last trunk segments (Brena and Akam, 2013). In another study, *Sm-Delta* and *Sm-Notch* expression were observed over the course of segmentation in correlation with expression of the pair-rule gene homologues *Sm-eve* and *Sm-hairy*. It could be shown that the dynamic expression patterns for all genes investigated, correlated in terms of periodicity in the posterior. It was hence suggested, that the pair-rule genes examined, are regulated by Delta/Notch signaling during *Strigamia* segmentation, as it has been described in other arthropods (Chipman and Akam, 2008).

Taken together, the analysis of the regulation of the formation and function of the SAZ among several arthropods suggest that Wnt and Delta-Notch signaling regulated this process ancestrally, in a mechanism similar to the regulation of somitogenesis in vertebrates (Bolognesi et al., 2008; Chesebro et al., 2012; McGregor et al., 2008b; Oda et al., 2007; Stollewerk et al., 2003).
1.5. *Parasteatoda tepidariorum* as a model to study arthropod segmentation

1.5.1. Chelicerates

Chelicerates branch at the base of arthropods and therefore are the sister group to myriapods, crustaceans and hexapods (Giribet and Edgecombe, 2012; Giribet, 2005; Regier et al., 2010) (see fig. 9). The origin of chelicerates has been dated back to the Cambrian, over 500 million years ago using the fossil records and molecular data (Dunlop, 2010; Rota-Stabelli et al., 2013). The chelicerates can be divided into the euchelicerates and the pygnogonid sea spiders with both exhibiting a pair of chelicere and chelifore appendages, respectively (Dunlop and Arango, 2005; Weygoldt and Paulus, 1979) (see fig. 9).

![Figure 9 | Arthropod phylogeny.](image)

**Figure 9 | Arthropod phylogeny.** Within the arthropod phylogeny, chelicerates (pycnogonida and euchelicerata) branch at the base. Crustaceans and hexapods are the most derived groups and therefore at the top of the tree. The myriapods form a sister group to the insect/crustaceans clade. (Regier et al., 2010; Rota-Stabelli et al., 2011; Weygoldt and Paulus, 1979)

Owing to their phylogenetic position, chelicerates can contribute greatly to our understanding of ancestral arthropod features and providing a good reference
point for hypotheses about the molecular mechanisms of development and the body plan of the last common ancestor of bilateria (Schwager et al., 2015).

The genomes of the two-spotted spider mite (*Tetranychus urticae*), a scorpion (*Mesobuthus martensii*), three spiders (*Stegodyphus mimosarum*, *Acanthoscurria geniculate*, *Parasteatoda tepidariorum*) and a horseshoe crab species (*Limulus polyphemus*) have been sequenced and analysed (Cao et al., 2013; Grbic et al., 2011; Nossa et al., 2014; Sanggaard et al., 2014; Schwager et al., 2016, in prep.). Interestingly, large variation in the genome sizes, as well as differences in the predicted gene content have been observed among chelicerate genomes. It has been suggested that events like whole genome duplications, for example in *Limulus* (Nossa et al., 2014), or extensive gene loss, as found in *Tetranychus* (Grbic et al., 2011), are responsible for this variation among chelicerate genomes.

### 1.5.2. *Parasteatoda* the model organism

The basal phylogenetic position of chelicerates among arthropods (Regier et al., 2010; Rota-Stabelli et al., 2011), as well as the well described embryonic development (Anderson, 1973; Kanayama et al., 2010; Mittmann and Wolff, 2012), and the availability of molecular tools, have made the common house spider *Parasteatoda tepidariorum* a powerful model organism in the field of evolutionary developmental biology (Hilbrant et al., 2012; McGregor et al., 2008a).

*Parasteatoda* is a cobweb making spider native to the neotropics, but is now a ubiquitous species. Females make cocoons containing up to 400 embryos.
about every 5 days, all year around under laboratory conditions. Due to the short fertilization process, which takes about three minutes, embryos develop synchronously within one cocoon, which is particularly advantageous for developmental studies. Embryos of all embryonic stages can be fixed and used for in situ hybridisation and antibody staining to study mRNA and protein expression, respectively (Prpic et al., 2008a; Prpic et al., 2008c). Furthermore, gene function can be studied in *Parasteatoda* with RNA interference. Double-stranded RNA (dsRNA) injected into adult females results in the embryos in several cocoons exhibiting a knockdown effect (Prpic et al., 2008b). While injecting a single cell of an embryo at 16- and 32-cell stages with dsRNA generates clones of cells lacking gene function (Kanayama et al., 2010). Moreover the *Parasteatoda* genome has been sequenced in addition to transcriptomic resources (Posnien et al., 2014; Schwager et al., in prep) and microRNA expression data (Leite et al., 2016).

1.5.3. *Parasteatoda* development

Upon fertilization, the first synchronized nuclear divisions take place in the center of the spherical egg. The energids start to migrate towards the periphery after about five divisions and cellularise at around the 16-cell stage (see fig. 10 A). Cells divide further and aggregate at one hemisphere to form the blastoderm (see fig. 10 A). The blastopore forms in the center of the germ disc upon gastrulation and invagination processes occur. After blastopore closure, the cumulus, a cluster of mesenchymal cells in the center of the germ disc, migrate underneath the ectodermal cell layer towards the rim of the germ
disc (see fig. 10). This process specifies the DV axis and initiates the transformation from a germ disc to a germ band (see fig. 10). The embryo then acquires a fan-like shape, whereby the caudal lobe forms from the central region of the previous germ disc. The sequential addition of opisthosomal segments from the posterior SAZ follows, and the nervous system and appendages begin to form along the AP axis (see fig. 10 A). At late stages of embryonic development, inversion occurs during which the embryo encloses the yolk and internal organs like the heart, digestive tract, and brain develop (see fig. 10 A). Embryonic development until hatching takes about 8 days and then it takes another 12 weeks for the spiderlings to develop to adulthood at 25 °C (Anderson, 1973; Kanayama et al., 2010; Mittmann and Wolff, 2012; Schwager et al., 2015) (see fig. 10 A, B).
Figure 10 | *Parasteatoda* embryonic development. (A) At stage 2 cellularisation is complete and the germ disc including the primary thickening (pt) in the center forms at stage 4. At stage 8, the germ band with the segment addition zone (SAZ) has developed. At stage 10 the germ band has elongated and the limbs are becoming morphologically visible. Inversion occurs between stage 10 – 13, marked by the internalisation of yolk. At stage 14 the embryo is fully developed with a clear constriction (arrowhead) between prosoma (Pro) and opisthosoma (Op). After hatching the postembryo develops into the 1st instar, which exits the cocoon. The 3rd instar represents a free-foraging instar stage. (B) Adult female (♀) spider at the top, male (♂) adult spider at the bottom. Picture taken from (Hilbrant et al., 2012; Rota-Stabelli et al., 2011). Staging was carried out after (Mittmann and Wolff, 2012; Regier et al., 2010) and the picture was modified from (Hilbrant et al., 2012).
1.6. Aims of the thesis

Further investigating the GRN of posterior development in the basally branching arthropod *Parasteatoda tepidariorum* will not only help elucidate the mechanisms involved in regulating short germ segmentation in arthropods and the evolution of these processes in arthropods and other metazoans. Therefore, I set out to address the following questions in my PhD thesis:

1. **Investigating the dynamic interactions of Delta-Notch and Wnt signaling in Parasteatoda**
   - Characterising the expression and role of the receptor *Pt-Notch*
   - Investigating the interactions between Delta/Notch and Wnt signaling
   - Investigating the *Pt-Delta* protein localisation using CRISPR

2. **Characterisation of downstream targets of Wnt and Delta-Notch signaling**
   - Characterising the expression and role of *Pt-caudal*
   - Analysing expression patterns of pair-rule orthologs

3. **Characterisation of the GRN underlying posterior segmentation**
   - Examining the effect of Delta-Notch and Wnt signaling on the downstream factors *Pt-eve* and *Pt-run-1*
   - Understanding the regulatory impact of *Pt-cad* on the pair-rule genes orthologue *Pt-eve* and *Pt-run-1*
- Investigating interactions between pair-rule gene orthologues in *Parasteatoda*

4. Analysis of Frizzled receptors in *Parasteatoda*
- Identification of Frizzled receptor genes
- Characterisation of the expression of Frizzled receptor genes during embryonic development
2. Materials & Methods

2.1. Spider culture, Embryo collection, fixation and staging

The spider culture at Oxford Brookes was initially founded with spiders from Göttingen (Germany). Adult spiders were kept separately in *Drosophila* vials (175 mm multipurpose container, Greiner) with coconut husk (generic from pet shop) and fed with banded crickets (size 2, Livefoods direct) twice a week. Mated females produce a cocoon every 3-5 days, whereby only up to 5 consecutive cocoons were taken from one female to ensure good quality of the embryos. The cocoons were collected daily and kept separately from the mother in petri dishes, with a piece of Whatman paper dampened with tap water to keep the spiderlings in a humid environment. Starting from a few days after hatching, the spiderlings were fed with vestigial flies twice a week. When the juvenile spiders reached the body size of about 5 mm, they were transferred to separate vials. The spider culture was kept at 25°C and embryos were fixed as described in Akiyama-Oda and Oda (2003). Embryos were staged according to Mittmann and Wolff (2012).

2.2. General molecular biology

2.2.1. RNA extraction and cDNA synthesis

Embryos of the stages 5 to 9 (see fig. 10) were collected and stored at -80°C. From a mix of those stages total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen). cDNA was synthesised from total RNA with the QuantiTect Reverse Transcription Kit (Qiagen).
2.2.2. PCR

Gene-specific cDNA fragments for in situ probe generation of dsRNA preparation were amplified with primers designed with Primer3 (http://primer3.ut.ee) using the OneTaq 2x Master Mix (New England Biolab, NEB). The primers were only designed to cover a sequence of the coding region of the genes examined.

25 µl reaction mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>forward primer (10 mM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>reverse primer (10 mM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>template DNA</td>
<td>variable</td>
</tr>
<tr>
<td>OneTaq 2x MasterMix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to 25 µl</td>
</tr>
</tbody>
</table>

PCR program

- initial denaturation: 94°C 30 s
- denaturation: 94°C 30 s
- annealing: 45-68°C 30 s
- extension: 68°C 1 min/kb
- final extension: 68°C 5 min
- final hold: 10°C

x 35 cycles

Parasteatoda Frizzled sequences are available from the Assembled Searchable Giant Arthropod Read Database ASGARD: Pt-fz1 (Locus 7239), Pt-fz2 (Locus 1), Pt-fz 4-1 (Locus 7239) and Pt-fz 4-2 (Locus 2608) (Zeng and Extavour, 2012). Pt-Sfrp, Pt-cad, Pt-eve, Pt-DI, Pt-run-1, Pt-odd, Pt-slp and Pt-opa sequences were obtained from the Parasteatoda transcriptome (Posnien et al., 2014).
### Materials & Methods

<table>
<thead>
<tr>
<th>gene name</th>
<th>product size</th>
<th>forward primer</th>
<th>reverse primer</th>
<th>method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt-fz1</td>
<td>980 bp</td>
<td>CCCGAACATGGATTGGTGTTG</td>
<td>CTTTTGGCAGAACATGCTGGATGG</td>
<td>ISH</td>
</tr>
<tr>
<td>Pt-fz2</td>
<td>589 bp</td>
<td>TCCCTATGGTTTGCCGCT</td>
<td>ATCGGGCTTCTTTCTGGATGG</td>
<td>ISH</td>
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<tr>
<td>Pt-fz4-1</td>
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<td>ISH</td>
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<tr>
<td>Pt-fz4-2</td>
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<td>CATTTTCTGAAGGCTGTCGAGGA</td>
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</tr>
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<td>Pt-eve</td>
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<td>TGTGGAGAATGGTGCTGGT</td>
<td>ISH, RNAi</td>
</tr>
<tr>
<td>Pt-cad</td>
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<td>ISH</td>
</tr>
<tr>
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<td>456 bp</td>
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</tr>
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<td>Pt-cad F2</td>
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<td>TCAATGAGTACATTATACTATTT</td>
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<tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Pt-N F2</td>
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<td>TCTCTCTTGAGACGGACGTGCA</td>
<td>RNAi</td>
</tr>
<tr>
<td>Pt-run-1</td>
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<td></td>
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<td>RNAi</td>
</tr>
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<td>TCAGTATGCTTCCATAGACCT</td>
<td>RNAi</td>
</tr>
<tr>
<td>Pt-odd</td>
<td>912 bp</td>
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<td>ISH</td>
</tr>
<tr>
<td>Pt-opa</td>
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<td>CAGAATCCAGCGATGCGCA</td>
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<td>ISH</td>
</tr>
<tr>
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<td></td>
</tr>
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<td>Pt-slp</td>
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<td>ATCCGCGCCAGAATCCAGAAA</td>
<td>TCAATCCTTGGAGAATGTCACCA</td>
<td>ISH</td>
</tr>
</tbody>
</table>

**Table 1** Primer sequences for all gene used in in situ and dsRNA experiments. Gene name; size of the product generated with the respective primer pair; sequence of the forward and reverse primer in 5’-3’ orientation; method (ISH, in situ hybridisation; RNAi, RNA interference) the fragment was used for. All primers were designed to cover a fragment of the coding region of the respective gene.

The PCR product was loaded on a 1% agarose gel and the specific band was purified from the gel using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel).

#### 2.2.3. Cloning

Subsequently, the PCR product was ligated into the TOPO PCR4 vector using the TOPO TA kit (cloning of Taq-polymerase amplified PCR products, Invitrogen), according to the manufacturers guidelines:
The ligation was transformed into OneShot TOP10 chemically competent cells (Invitrogen) according to the standard heat shock transformation protocol:

- TOP10 cells are thawed on ice
- 2 µl of the TOPO cloning reaction is added to the cells, mixed gently and incubated on ice for up to 30 min
- TOP10 cells are heat-shocked in a water bath at 42°C for 30 sec and immediately transferred to ice
- 250 µl S.O.C. medium (Invitrogen) is added and incubated in a shaking incubator at 200 rpm, at 37 °C for 1 hour
- 50 µl of the transformed cells were plated on Lennox Broth (LB) plates with ampicillin (100 µg/ml) and incubated at 37 °C over night

2.2.4. Colony PCR and overnight cultures

Colonies were then picked for PCR using the OneTaq 2x Master Mix (NEB) and plated and numbered on a Lennox Broth (LB) plates with ampicillin (100 µg/ml), to track the colonies. The PCR product was loaded on a 1% agarose gel and checked for the correct product size. Colonies with the correct insert size were grown in 5 ml liquid LB cultures with ampicillin (100 µg/ml) at 37 °C in a shaking incubator over night.

Plasmid mini preparations were made from the liquid cultures using the EZNA Plasmid Mini Kit I (VWR) according to the manufacturers guidelines and verified with Sanger sequencing (Eurofins).

**reaction mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>0.5 – 4 µl</td>
</tr>
<tr>
<td>salt solution</td>
<td>1 µl</td>
</tr>
<tr>
<td>water</td>
<td>up to 5 µl</td>
</tr>
<tr>
<td>TOPO vector</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

mix the reaction gently and incubate at room temperature for 5 minutes for 5 minutes.
2.2.5. Sequencing

Plasmids and PCR products were sequenced with the value-read service of Eurofins Genomics (Germany). Alignments of sequences and in silico design of constructs were done in SnapGene Version 2.8.

2.2.6. In situ probe synthesis

RNA probes were labelled with Digoxigenin (DIG; Roche) and detected with an alkaline phosphatase conjugated anti-DIG antibody (Fab fragments, Roche) using the substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche), resulting in purple/blue staining.

For double in situ hybridisation, the second probe was labelled with fluorescein (Roche) and detected with an alkaline phosphatase conjugated anti-fluorescein antibody (Fab fragments, Roche) and INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)/BCIP (Roche), resulting in orange staining.

2.3. In situ hybridisation protocol

In situ hybridisations were carried out according to the whole-mount protocol for spiders (Prpic et al., 2008d) with minor modifications. In order to decrease the background in the staining reaction, the anti-DIG and anti-fluorescein antibodies were pre-absorbed over night at 4°C with stage 6 to 8.2 embryos. Note that in situ hybridisation staining reactions on control and experimental (RNAi) embryos were carried out for the same time. For double in situ
hybridisations, the first staining reaction was stopped by incubating the samples for 15 minutes at 65°C with inactivation buffer (50 ml hybridisation buffer B, 0.1 ml 10% Tween-20, 1.5 ml 10% SDS). The embryos were then washed twice with PBS-T for 15 minutes and twice for 20 minutes. Subsequently, the embryos were incubated in blocking solution for 30 minutes and then with the anti-Fluorescein antibody at a dilution of 1:2000 in blocking solution (Roche) for 3 hours. Nuclear staining was performed by incubation of embryos in 1 µg/ml 4-6-diamidino-2-phenylindol (DAPI) in PBS with 0.1% Tween-20 for 30 minutes and subsequently washed with PBS-T with 0.1% Tween-20 twice for 5 minutes.

2.4. RNAi interference

2.4.1. Double stranded RNA preparation

To generate double stranded RNA (dsRNA), PCR fragments (for primer sequences and fragment sizes see table 1) of the coding regions were amplified from plasmids using universal primers, which both contain a 5’ T7 promoter binding site (Fwd T7 5’-TAA TAC GAC TCA CTA TAG GG-3’, Rev T7/T3 5’-TAA TAC GAC TCA CTA TAG GGA ATT AAC CCT CAC TAA AGG GA-3’). The introduction of the T7 promoter sequence on the antisense strand, using the Rev T7/T3 primer, allows the in vitro transcription of both strands in one reaction.

The PCR was carried out as described in chapter 2.2.2. and the PCR product was purified from a 1% agarose gel using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel).
PCR products were used as templates for *in vitro* reverse transcription of both strands with the MegaScript T7 transcription kit (Invitrogen) according to the manufacturers guidelines. dsRNA was then generated by annealing the transcripts in a water bath starting at 95°C, and then slowly cooled down to room temperature. The dsRNA was then adjusted to a concentration of 1.5 to 2.0 µg/µl for injections.

### 2.4.2. Parental RNAi (pRNAi)

For each gene, at least three adult female spiders were injected according to the protocol by Akiyama-Oda and Oda (2006). 2 µl dsRNA was injected into the opisthosoma of spiders at concentrations of 1.5-2.0 µg/µl every two to three days up to a total of five injections. The injected spiders were mated after the second injection. Embryos from injected spiders were fixed for gene expression and phenotypic analyses two and four days after egg laying approximating to stages 6 to 9.2. Embryos from GFP injected control females were generated and treated as described above.

### 2.4.3. Embryonic RNAi (eRNAi)

Embryonic injections were carried out as described in Kanayama et al. (2010) with minor changes (GC100F-10 capillaries, Harvard Apparatus; needle puller PC-10, Narishige or Femtotip II sterile injection capillary 0.5 µm, Eppendorf). Embryos were injected at the 8 or 16 cell stage with an injection mix composed of 10 µl Fluorescein isothiocyanate (FITC)-dextran (2 µg/µl, MW 10 000, Sigma), 10ul Biotin-dextran (2 µg/µl, MW 10 000, Sigma) and 5 µl
dsRNA (1.5 to 2.0 µg/µl) and fixed when they reached developmental stages 6 or 7. In order to visualise the clones of eRNAi cells, the co-injected Biotin-dextran was detected with the Vectastain ABC-AP kit, which was carried out according to the manufacturers protocol (Vector Laboratories) following the in situ hybridisation. At least 200 embryos were injected for each gene of interest to ensure that multiple independent clones were generated in the SAZ.

2.5. Synthesis and overexpression of capped mRNA

The pSP64-cad-eGFP-PolyA plasmid was generated by Christian Bonatto Paese. In order to synthesise capped mRNA, the pSP64-cad-eGFP-PolyA plasmid was linearized with NheI (Promega) according to the manufacturers protocol, the respective band cut from a 1% agarose gel and purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). The linearized plasmid was used as a template for the SP6 transcription reaction with mMESSAGE mMACHINE® SP6 Transcription Kit (Ambion™) following the manufacturer’s instructions. Capped mRNAs was injected as described by Kanayama et al., 2010.

2.6. CRISPR construct generation for the C-terminal tagging of Pt-DI

The CRISPR/Cas9 system can be used to either introduce mutations through error-prone non-homologous end joining (NHEJ) or to insert a sequence of interest at a specific locus through homologous recombination (HR) (Baena-
Lopez et al., 2013; Bassett et al., 2013; Gratz et al., 2014). In order to introduce double strand breaks (DSB) at a given locus in the genome, a synthetic guide RNA (sgRNA) binds the Cas9 endonuclease and directs the complex to the target sequence through complementary base pairing. The sgRNA contains the complementary target sequence of about 20 nucleotides at the 5’ end, followed by a protospacer adjacent motif (PAM), which is necessary for the Cas9 endonuclease activity and a loop structure at the 3’ end for the recognition by the Cas9 (see fig. 11).

For the C-terminal tagging of Pt-Delta, the 3’ and 5’ homology arm (3’HA, 5’HA) were cloned into the multiple cloning sites (MCS) of the pHD-dsRed plasmid, which contains the fluorescent marker cassette dsRed (Discosoma sp. red fluorescent protein) and the translation termination signal SV40 (simian vacuolating virus 40) (see fig. 12). The pHD-dsRed plasmid was gifted to Pedro Gaspar.
The 5' homology arm (HA) contains 1 kb upstream of the Pt-Dl stop codon (Pt-Dl coding sequence, excluding the stop codon TAA) and the 3'HA contains 1 kb of the intergenic region (no Augustus gene prediction) upstream of the Pt-Dl coding region (see fig. 12).

To generate the C-terminal fusion of Pt-Dl with the dsRed cassette, a 314 bp region between the MCS for the 5'HA and the dsRed cassette had to be excised from the original plasmid and an AarI recognition site was inserted 5' of the dsRed cassette. Primers were designed to amplify the vector backbone from the 3' end of the MCS of the 5'HA (pHD_dsRed_3'HA_AarI_fwd: CACGCACCTGCAATTGCCGCGATGGCCTCCTCCGAGGACGTCA, pHD_dsRed_3'HA_AarI_rev: TGCATATGTCCGCGGCCGCTAG) up to the start of the dsRed cassette, including the AarI recognition sequence. Two non-polar amino acids (Alanin, GCC/GCG) were added between the AarI site and the dsRed start codon, to maintain the reading frame.

Figure 12 | sgRNA priming site. 55bp region of the 5’ homology arm region (Pt-Dl CDS, Pt-Dl 3’UTR containing the sgRNA priming site (sgRNA, blue bar). The Cas9 cut site is located 3 bases upstream of the PAM sequence (grey bat), indicated by a black arrow. The endogenous Pt-Dl stop codon (TAA) is marked with a red bar. The seed region (turquoise bar) is crucial for the recognition by the sgRNA and must not contain PCR amplification errors.
The vector backbone was amplified from the original plasmid using the Q5 High Fidelity DNA Polymerase, according to the protocol (NEB).

**25 µl reaction mix**

<table>
<thead>
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<th>Volume</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>10 µM dNTPs</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>forward primer (10 µM)</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>reverse primer (10 µM)</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>template DNA</td>
<td>variable</td>
</tr>
<tr>
<td>Q5 High-Fidelity</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA Polymerase</td>
<td>to 25 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
</tr>
</tbody>
</table>

**PCR program**

- initial denaturation 98°C 30 s
- denaturation 98°C 30 s
- annealing 50-72°C 30 s
- extension 72°C 20-30 sec/kb
- final extension 72°C 2 min
- final hold 10°C

x 35 cycles

The PCR product was loaded on a 1% agarose gel and the specific band was purified from the gel using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel).

### 2.6.1. Q5 Site-directed mutagenesis

The purified PCR product was treated with the Kinase-Ligase-DpnI enzyme mix according to the manufacturers protocol (Q5 Site-directed Mutagenesis Kit, NEB), to eliminate possible contamination with the template plasmid and circularisation of the PCR product at room temperature. Subsequently, the circularized product was transformed into chemically competent TOP10 cells (Invitrogen) according to the standard heat shock transformation protocol.
(also see chapter 2.2.3), plated on LB plates with ampicillin (100 µg/ml) and incubated at 37 °C over night.

Following, colony PCR was carried out and liquid cultures of positive clones were generated (see chapter 2.2.4). Plasmid preparations were performed and sent for Sanger sequencing (see chapter 2.2.5).

To enable the insertion of the 3’ and 5’HA, primers were designed containing the specific and the restriction enzyme recognition sequence:

3’HA_SapI_fwd: CACGGCTCTTCTCATttgctataagaatatagcctgtgatctag,
3’HA_SapI_rev: CACGGCTCTTCCGGAcatcgggtgattttttggatttaaaatcaagg,
5’HA_AarI_fwd: CACGCACCTGCCACATCGCaatccctgccttaatgg,
5’HA_AarI_rev: CACGCACCTGCGTGTCGGCccttttgctcgatgctatatttga

The 3’ and 5’HA regions were amplified from genomic DNA (obtained from Daniel Leite) with the Q5 High Fidelity DNA polymerase (NEB) and purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Subsequently, the mutagenized pHD-dsRed plasmid (introduced AarI sites) and the 5’ HA fragment were digested with AarI (ThermoFisher) according to the manufacturers protocol at 37 °C for 4 hours. The 5’ HA fragment was purified with the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Afterwards, the 5’ HA fragment was ligated into the digested pHD-dsRed plasmid with the T4 DNA Ligase system (Promega) according to the manufacturers protocol at 15°C over night (see fig. 13). The ligated product was transformed into chemically competent TOP10 cells (Invitrogen) according to the standard heat shock transformation protocol, plated on
Materials & Methods

Lennox Broth (LB) plates with ampicillin (100 µg/ml) and incubated at 37 °C over night.

Afterwards colony PCR was performed and positive colonies cultured in 5ml liquid LB cultures with ampicillin (100 µg/ml) at 37 °C in a shaking incubator over night. Plasmid mine preparations were made from the liquid cultures using the EZNA Plasmid Mini Kit I (VWR) and verified with Sanger sequencing. The protocol for restriction and ligation was repeated for the 3’HA (see fig. 13). The final plasmid was verified with Sanger Sequencing and the LB cultures were used to prepare a glycerol stock for long-term storage at -80°C.

**Figure 13 | Cloning of the 5’ and 3’ homology arms (5’/3’HA).** The pH-D-dsRed plasmid contains a 5’ multiple cloning site (MCS) flanked by AarI restriction sites (light blue), followed by the dsRed (red) and SV40 (yellow) cassette, adjacent to a 3’ MCS flanked with SapI sites (orange). The 5’HA PCR product (light blue) contains AarI restriction cut sites at the 5’ and 3’ end. First, the plasmid and the 5’HA PCR product were digested with AarI, respectively and then ligated with the T4 ligase (1). In the second step, the newly generated plasmid and the 3’HA PCR product (light blue) were digested with SapI and further ligated with the T4 ligase (2). The obtained plasmid comprises the 5’HA, in frame with dsRed and SV40 and the 3’HA (3).
2.6.2. Short guide RNA (sgRNA) design and synthesis

The sgRNA guides the Cas9 endonuclease to the region of interest and the precise cut site is determined by the PAM sequence (NGG). The forward primer, containing the cut site close to the stop codon and the PAM sequence, for the amplification of the *Pt-Dl*-sgRNA, were predicted using the online tool http://crispr.mit.edu. In order to synthesise the sgRNA with a T7 polymerase *in vitro* transcription reaction, a T7 promoter sequence was added 5’ of the gene specific sequence. 3’ of the gene specific sequence, an overlap with the sgRNA reverse primer was added (5’

GAAATTAATACGACTCACTATAGGN_{18-20nt} gene specific

sequence GTTTTAGAGCTAGAAATAGC 3’; the font indicates the overlapping region with the sgRNA reverse primer). The sgRNA reverse primer (sgRNA_rev AAAAGCACCAGCTCGGTGCCACTTTTCAGTTGATAACGG ACTAGCCTTATTTTAACCTTGCTATTTCTAGCTCTAAAAC; the bold part of the sequence indicates the overlapping region with the sgRNA forward primer) is a universal primer, containing the stem loop structure for the incorporation of the sgRNA into the Cas9 complex.

The *Pt-Dl*-sgRNA template was amplified using the Phusion polymerase (NEB) as follows:

<table>
<thead>
<tr>
<th>reaction mix</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>98°C 30 s</td>
</tr>
<tr>
<td>5x HF buffer</td>
<td>98°C 10 s</td>
</tr>
<tr>
<td>10 µM dNTPs</td>
<td>60°C 30 s</td>
</tr>
<tr>
<td>Phusion DNA polymerase</td>
<td>72°C 15 s</td>
</tr>
<tr>
<td>sgRNA_fwd primer (10 µM)</td>
<td>72°C 10 min</td>
</tr>
<tr>
<td>sgRNA_rev primer (10 µM)</td>
<td>4°C hold</td>
</tr>
<tr>
<td>67 µl</td>
<td></td>
</tr>
<tr>
<td>20 µl</td>
<td></td>
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<tr>
<td>2 µl</td>
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<td>1 µl</td>
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<td>5 µl</td>
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<tr>
<td>5 µl</td>
<td></td>
</tr>
<tr>
<td>100 µl</td>
<td></td>
</tr>
</tbody>
</table>
The PCR product was purified with the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel) purification kit and eluted in 30ml elution buffer. The PCR product was used as a template for *in vitro* transcription with the T7 MegaScript kit (Invitrogen, Life Technologies), according to the manufacturers protocol.

**reaction mix**

\[
\begin{align*}
6 \mu l & \text{nuclease free water} \\
2 \mu l & \text{ATP (75 mM)} \\
2 \mu l & \text{CTP (75 mM)} \\
2 \mu l & \text{GTP (75 mM)} \\
2 \mu l & \text{UTP (75 mM)} \\
2 \mu l & 10x \text{reaction buffer} \\
2 \mu l & \text{PCR product (150 ng/ml)} \\
2 \mu l & \text{enzyme mix (T7)} \\
\end{align*}
\]

\[20 \mu l\text{ incubate at }37^\circ C\text{ for 4h}\]

The reaction was incubated with 1µl TurboDNAse (included in the kit) at 37°C for 15 minutes. To stop the reaction 115µl nuclease free water and 15µl ammonium acetate stop solution were added.

The sgRNA was precipitated by adding 150µl phenol:chloroform:isoamyl alcohol (24:24:1), pH 7 and vortexing thoroughly for 30 seconds. After a spin cycle of 10000 g for 3 minutes at room temperature, the upper layer was transferred into a fresh tube and precipitated with 150µl isopropanol at -20°C for at least 15 minutes. After another spin cycle at 17000 g for at least 15 minutes at 4°C, the supernatant was discarded and the RNA pellet was washed with 500µl ethanol. The ethanol wash was repeated, the pellet dried briefly and resuspended in 30µl ddH₂O. The sgRNA was run on a gel and the concentration measured on a NanoVue spectrophotometer (GE lifesciences).
2.6.3. CRISPR injection protocol

The guidelines for the preparation of the CRISPR injection mix were obtained from Andrew Bassett (University of Oxford). The sgRNA, the Cas9 protein (also gifted from Andrew Bassett) and the donor plasmid were assembled as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Final Concentration</th>
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</thead>
<tbody>
<tr>
<td>water</td>
<td>15 ul</td>
</tr>
<tr>
<td>10x buffer</td>
<td>2 ul 1x</td>
</tr>
<tr>
<td>~1 ug/ul Cas9</td>
<td>1 ul 50 ng/ul</td>
</tr>
<tr>
<td>0.5 ug/ul sgRNA</td>
<td>1 ul 25 ng/ul</td>
</tr>
</tbody>
</table>

**10x buffer**

200 mM HEPES pH 7.5 200 ul (1 M)
1000 mM KCl 500 ul (2 M)
25 mM MgCl2 12.5 ul (2 M)
5% glycerol 100 ul (50%)
1 mM EDTA 2 ul (0.5 M)
5 mM DTT 50 ul (0.1 M)
Water 135.5 ul

1 ml

The injection mix was pre-incubate at 37°C for 10 min. Afterwards the donor plasmid was added at a concentration of 500 ng/ul DNA.

2.7. Data documentation

Embryos were imaged using a Leica fluorescence stereomicroscope equipped with a Jenoptik ProgRes C3 digital camera. Brightfield and UV channel images were merged using Adobe Photoshop CS6, which was also used for linear corrections of brightness, contrast, and colour values. Images for the Pt-cad overexpression experiment were taken with a Zeiss Axio Zoom V16 stereomicroscope, equipped with an Axiocam 506 monoand a colour digital camera.
2.8. Protein sequence alignments

Blastp (www.blast.ncbi.nlm.nih.gov) was used to identify sequence conservation with the Pt-Eve homeodomain and Pt-Sfrp frizzled-like CRD domain. Species with the highest conservation were aligned manually.

2.9. Phylogenetic analysis

The nucleotide sequences of all species included were obtained from NCBI (http://www.ncbi.nlm.nih.gov). The phylogenetic analysis was carried out using the “one click” method of the online tool “Phylogeny.fr: robust phylogenetic analysis for the non-specialist” (Dereeper et al., 2008). The program uses MUSCLE for the sequence alignment (Edgar, 2004), GBlocks for sequence curation (Castresana, 2000), PhylML for the maximum-likelihood phylogeny analysis (Guindon et al., 2010) and the TreeDyn software for the graphical output (Chevenet et al., 2006).
3 Results Chapter 1:
Dynamic interactions between Pt-Dl, Pt-N and Pt-Wnt8 regulate posterior segmentation in Parasteatoda

It has been shown previously that both Wnt and Delta-Notch signaling are crucial for the formation of the SAZ and the sequential formation of segments from this tissue in Parasteatoda (McGregor et al., 2008b; Oda et al., 2007). Indeed, it is likely that this involves interplay between these two pathways, because it was shown that in Pt-Wnt8 RNAi knockdown embryos Pt-Dl expression is established normally, but subsequently fails to clear and persists in the posterior (McGregor et al., 2008b). This suggests that Pt-Wnt8 is necessary for dynamic Pt-Dl expression. However, it is unclear if Pt-Dl also regulates Pt-Wnt8. Therefore I investigated the expression, roles and interactions between Delta-Notch and Wnt8 signaling during posterior development in Parasteatoda in more detail.

3.1 The role of Pt-Dl in posterior development in Parasteatoda

As previously reported, Pt-Dl expression commences early in embryonic development at mid stage 4 in a few ectoderm cells around the rim of the germ disc and then in future mesodermal cells in the centre of the germ disc at a slightly later stage (Oda et al., 2007). During stage 6, after Pt-Dl expression has cleared from posterior SAZ cells, this gene is expressed in a salt and pepper pattern adjacent to a more diffuse posterior domain in anterior SAZ cells (Oda
et al., 2007) (see fig. 14 A). In contrast, *Pt-Wnt8* expression is weaker in anterior SAZ cells, where it overlaps with *Pt-Dl* expression, compared to the stronger expression of *Pt-Wnt8* detected in posterior SAZ cells (see fig. 14 B). Although this aspect of *Pt-Wnt8* expression was noticed previously, it was suggested it might be a gradient in expression rather than a domain of where *Pt-Wnt8* expression is specifically down regulated (McGregor et al., 2008b).

To investigate the role of *Pt-Dl* further, I knocked down the expression of this gene using pRNAi. This treatment resulted in the loss of *Pt-Wnt8* expression in the posterior of the SAZ, but conversely gave rise to stronger *Pt-Wnt8* expression in anterior SAZ cells (see fig. 14 C). This suggests that *Pt-Dl* is required to activate *Pt-Wnt8* expression in posterior SAZ cells but then when *Pt-Dl* expression reaches the anterior SAZ cells it is involved in down-regulating *Pt-Wnt8* possibly to facilitate the formation of segments from this tissue.
Results

3.2 The role of Pt-N in posterior development in Parasteatoda

To study Delta-Notch signaling in posterior development in Parasteatoda further, I then characterised the expression of Pt-N in the embryos of this spider. The expression of Pt-N commences at stage 5 in a two to three cell wide band around the germ disc (see fig. 15 A). Slightly later, a second ring of Pt-N expression appears more centrally (data not shown). After the dorsal opening and the formation of the germ band at stage 6, diffuse expression appears in...
the posterior SAZ (see fig. 15 B). The posterior SAZ expression then becomes stronger and the anterior domain forms a broad band at stage 7 (see fig. 15 C).

*Pt-Delta*, as well as *Pt-Notch* expression commence at the rim of the germ disc at a similar stage (around stage 5) (see fig. 15 A, Oda et al., 2007). *Pt-Delta* initially clears from the posterior at stage 6, however, *Pt-N* is expressed diffusely in the whole SAZ area at stage 6 and 7 (see fig. 15 B, C). Furthermore, *Pt-Delta* and *Pt-N* expression resolve into anterior stripes, although the *Pt-N* stripe appears uniform, whereas *Pt-Delta* expression displays a diffuse stripe adjacent to a more anterior stripe that has a salt and pepper pattern (see fig. 14 A, fig 15 B, C).

It has been reported previously that pRNAi against *Pt-N* has a similar effect to the knockdown of *Pt-Dl* in early *Parasteatoda* embryos and that the expression of *Pt-Dl* is disrupted in *Pt-N* RNAi embryos (Oda et al., 2007). Therefore, I next tested if *Pt-N* expression was reciprocally regulated by *Pt-Dl*. pRNAi against *Pt-Dl* leads to the loss of *Pt-N* expression in the anterior but stronger *Pt-N* expression in the posterior SAZ (see fig. 15 D). Thus the RNAi results suggest that *Pt-N* might inhibit *Pt-Delta* in posterior cells and activate its expression in the anterior of the SAZ, reminiscent of the effect of *Pt-Dl* on *Pt-Wnt8* (see fig. 14).
Results

To determine if Pt-Wnt8 is also regulated by Pt-N, I next examined Pt-Wnt8 expression in Pt-N pRNAi embryos. I found that Pt-Wnt8 also requires Pt-N (see fig. 14 B), reminiscent of the effect of Pt-Dl on Pt-Wnt8 expression (see fig. 16 C). More precisely, Pt-Wnt8 expression is restricted to an area in the posterior where the cell number is increased as a consequence of the Pt-N knockdown (Oda et al., 2007). These findings confirm that Delta-Notch signaling is necessary to first activate Pt-Wnt8 expression in posterior SAZ cells during stage 5, but subsequently down-regulates Pt-Wnt8 in anterior SAZ cells, possibly to facilitate the formation of segments from this tissue (see fig 15 and fig. 16).

Figure 15 | Pt-N expression and the effect of Pt-Dl on Pt-N. In wild-type embryos Pt-N is expressed in a 2-3 cells wide band around the rim of the germ disc at stage 5 (A). At stage 6, Pt-N is expressed in a salt and pepper pattern in the posterior and in a stripe domain in the anterior SAZ (B). Later at stage 7, Pt-N is expressed in the posterior SAZ and in a broad domain in the anterior portion of the germ band (C). Expression of Pt-N is lost in the anterior and is strongly expressed in the posterior SAZ in Pt-Dl parental RNAi embryos at that stage (D). Images A'-D'; show fluorescent staining with the nuclear marker DAPI of the respective bright field images A-D. A shows a germ disc stage embryo, panels B-D show posterior views of whole mount embryo with ventral oriented to the left.
3.3 Investigating Pt-Dl protein localisation in vivo using CRISPR

Many previous studies examined the function and interactions of components regulating segmentation in arthropods only at the mRNA level (Chesebro et al., 2012; Chipman and Akam, 2008; Choe et al., 2006; McGregor et al., 2008b; Pueyo et al., 2008; Sarrazin et al., 2012; Stollewerk et al., 2003). However, expression pattern analysis and RNAi knockdown entail the caveat that they
might not convey a complete picture, because the expression levels and the location of the protein is likely to make a significant contribution to our understanding of gene function and the underlying molecular mechanisms (Gilles and Averof, 2014). Thus, it was an objective of this thesis to try to establish a different experimental approach to investigate the localization of GRN proteins that appear to exhibit dynamic expression throughout posterior development in Parasteatoda. In particular, Pt-DI represents an interesting candidate for this study because its expression changes in association with the formation of posterior segments in the spider (Oda et al., 2007).

The Clustered Regular Interspersed Repeats / Caspase9 (CRISPR/Cas9) - genome editing method utilises modified components of a bacterial defence mechanism to introduce nucleotide specific double strand breaks (Bassett and Liu, 2014; Bhaya et al., 2011; Sander and Joung, 2014). CRISPR/Cas9 induced mutagenesis has already been successfully used for genome editing in various model organisms, including several arthropods (Auer et al., 2014; Bassett et al., 2013; Friedland et al., 2013; Gilles et al., 2015; Kistler et al., 2015; Nakanishi et al., 2014; Nakayama et al., 2013; Wei et al., 2014). In Parasteatoda, the knock-in of a fluorescent reporter via CRISPR/Cas9 at the Pt-Delta locus would enable the tracking of Pt-Delta protein at any stage of development in live embryos. Thereby, dynamic protein expression in the SAZ and the forming segments could be visualized throughout segmentation to further investigate the activity of a putative molecular clock regulating segmentation in Parasteatoda. In order to visualize Pt-Delta protein expression in vivo, a plasmid for the fusion of the fluorescent marker red fluorescent protein (dsRed) in frame with the Pt-Delta
coding region was generated. As the C-terminal tagging of the Delta protein with a fluorescent marker has been shown to be functional in *Drosophila* (Hagedorn et al., 2006), the sgRNA was designed against 20 nucleotides upstream of the *Pt-Delta* stop codon. This design ensures the seamless transcriptional transition of the *Pt-Delta* transcript to dsRed and thereby generates the C-terminal tagging of the *Pt-Delta* protein. The constructs also comprises the SV40 polyadenylation signal to ensure transcriptional termination (Wu and Alwine, 2004) (see fig 12).

I then injected 670 embryos with the *Pt-Dl-CRISPR* construct together with the fluorescent marker FITC, to track the clones *in vivo* (see chapter 2.6.3) and checked for a fluorescent signal every 24 hours for up to 4 consecutive days. The embryos displayed a very high survival rate of over 90% and I could detect the fluorescent dye FITC in somatic clones (see fig. 17), however, no dsRed signal could be detected at any of the time points observed.

![Fig.17](image-url) **Fig.17 | Pt-Dl::dsRed knock-in using CRISPR.** Panels A-C show the same embryo at stage 5. Panel A shows a bright field image of the injected embryo. The FITC signal in panel B visualizes the clone of cells comprising the injection mix. Panel C shows that no dsRed signal could be detected in the respective clone area.
This suggests that the integration of the \textit{Pt-Dl-CRISPR} construct was not successful for various methodological reasons, which will be discussed in the following. Furthermore the integration of the \textit{Pt-Dl-CRISPR} construct requires HR, which has been shown to be less efficient than NHEJ (Cong et al., 2013; Platt et al., 2014; Wang et al., 2013), hence an increase in the number of injected embryos, might enhance the probability of a positive result.

### 3.4 Discussion

#### 3.4.1 The role of \textit{Pt-N} and \textit{Pt-Dl} in the posterior of \textit{Parasteatoda}

In this work I showed that \textit{Pt-Dl} is required for the activation of \textit{Pt-Wnt8} in the posterior, but represses its expression in the anterior SAZ (Schonauer et al., 2016). This suggests that \textit{Pt-Dl} facilitates the expression of \textit{Pt-Wnt8} and that Delta-Notch signaling has a dual effect on \textit{Pt-Wnt8} expression in the SAZ (Schonauer et al., 2016). Moreover, it was previously shown that that \textit{Pt-Wnt8} is required to maintain a pool of cells in the posterior SAZ of \textit{Parasteatoda} and regulate cyclical expression of \textit{Pt-Dl} (McGregor et al., 2008b). Therefore, these complex regulatory interactions of Delta-Notch and \textit{Pt-Wnt8} in the posterior of \textit{Parasteatoda} embryos suggests that the formation and maintenance of the SAZ and the subsequent formation of segments from this tissue requires a functional compartmentalisation of the SAZ. This is consistent with findings in other arthropods (Brena and Akam, 2013; Chesebro et al., 2013).

In the centipede \textit{Strigamia}, expression analysis showed that \textit{Sm-Dl} and \textit{Sm-cad} are expressed out of phase with each other: the initial double segmental pattern in the posterior disc, a population of undifferentiated cells from which the
results

segments form, as well as the segmental expression in the forming trunk segments do not overlap between those two genes (Brena and Akam, 2013; Chipman et al., 2004).

Furthermore, study of the cockroach has shown that a positive feedback loop of \textit{Pa-Wnt1} and \textit{Pa-Dl} in the posterior in conjunction with \textit{Pa-cad} repressing \textit{Pa-Dl} in the more anterior region of the growth zone (GZ), are responsible for oscillating gene expression passing through the GZ (Chesebro et al., 2013). This functional subdivision of the \textit{Periplaneta} GZ maintains the GZ cells in an undifferentiated state and ensures stimuli of differentiation at regular intervals at the anterior (Chesebro et al., 2013).

Therefore studies of \textit{Parasteatoda} and other arthropods suggest that the SAZ can be subdivided into a posterior region that maintains a pool of undifferentiated cells and an anterior region, where the cells differentiate and nascent segments are forming. However, further work on spiders and other arthropods is needed to determine how these regions are specified and regulated and if common mechanisms are used across arthropods.

In \textit{Parasteatoda} \textit{Pt-Dl} and \textit{Pt-N} expression are both activated at a similar stage, in potentially overlapping domains at the rim of the germ disc and in the SAZ (see fig.15 A, Oda et al., 2007). However while \textit{Pt-Dl} clears from the posterior, \textit{Pt-N} expression remains diffusely in the whole SAZ area at the same stage (see fig. 14 A and fig. 15 B). Due to those differences in expression dynamics between \textit{Pt-Dl} and \textit{Pt-N}, it has been suggested that \textit{Pt-N} might be responsible for the maintenance of \textit{Pt-Wnt8} in the posterior and \textit{Pt-Dl} required for \textit{Pt-Wnt8}
repression in the anterior SAZ, whilst the overall effect on Pt-Wnt8 in both the Pt-DI and the Pt-N knockdown experiments appears similar (Schonauer et al., 2016).

Pt-DI and Pt-N, expression at least in the SAZ appears to overlap, however, Pt-N appears to be expressed more diffusely compared to the distinct Pt-DI expression (see fig.14 A and fig. 15 B). Together with the effect of Pt-DI and Pt-N expression and vice versa in reciprocal RNAi knockdown experiments, might be indicative of a regulatory feedback interaction in the Delta-Notch pathway (Oda et al., 2007; Schonauer et al., 2016).

Interestingly, it has been shown in Drosophila and in vertebrates that Delta and Notch undergo complex regulatory interactions, whereby Delta trans-activates Notch by binding to the receptor and reciprocally cis-inhibits Notch activity in the same cell (de Celis and Bray, 1997; del Alamo et al., 2011; Micchelli et al., 1997). This cis-inhibition mechanism increases the specificity of the signaling interaction, as the cell becomes unresponsive for signals from other cells, facilitating the generation of distinct cell fates in a pool of previously uniform neighbouring cells (del Alamo et al., 2011; Sprinzak et al., 2010). Furthermore it could be shown that fringe modulates ubiquitously expressed Notch in certain developmental compartments, like the dorsal of the Drosophila wing (Irvine, 1999; Takeuchi and Haltiwanger, 2010). This increases the affinity for Delta over the second ligand Serrate, contributing to border formation in developmental processes, like the dorsal and ventral compartment of the fly wing (Irvine, 1999; Takeuchi and Haltiwanger, 2010).
3.4.2 Investigation Pt-DI protein localisation using CRISPR

To better decipher if these or similar interactions between DI and N are involved in segment addition in spiders it is necessary to study their protein localization. Hence, I set out to label Pt-DI protein with the fluorescent marker dsRed using the genome editing method CRISPR. However, in the few attempts I undertook, I could not detect a fluorescent signal in the injected embryos. Nevertheless, the negative results for the CRISPR injections rely on a small number of injected embryos and only one concentration of sgRNA, Cas9 protein and donor plasmid has been tested. For the injection mix, I used the recommended concentrations for *Drosophila*. Given more time for these experiments I would inject different concentrations of sgRNA, because it has been shown to have a great influence on the targeting efficiency in *Drosophila* (Ren et al., 2014). Furthermore, I would co-inject multiple sgRNAs targeting the same locus (there are several sgRNA options available in the online prediction), as this has proven to increase the cutting efficiency of the Cas9 endonuclease in *Drosophila* (Ran et al., 2013) with the caveat that this could result in off target effects. While the basic requirements, like microinjection, are already available in *Parasteatoda*, it is now a matter of testing and optimizing various parameters in order to establish CRISPR successfully in the spider.
4 Results Chapter 2: 
Downstream targets of Wnt and Delta/Notch signaling

In previous studies of *Parasteatoda* posterior segmentation, it has been shown that *Pt-cad* and the mesodermal specification gene *twist* (*Pt-twi*) are expressed in the SAZ and the developing opisthosomal segments (Oda et al., 2007; Yamazaki et al., 2005). Furthermore, it could be shown that *Pt-cad* and *Pt-twi* are responsible for the correct formation of mesoderm and caudal ectoderm in the posterior of the developing embryo and that both factors are downstream targets of Wnt and Delta/Notch signaling in the spider (McGregor et al., 2008b; Oda et al., 2007; Yamazaki et al., 2005).

It has been concluded from *Pt-Dl* knockdown experiments, where *Pt-cad* expression is lost and caudal ectoderm is not able to form, that *Pt-cad* is downstream of Delta/Notch signaling. However, it has been suggested that *Pt-cad* cannot be directly activated by *Pt-Dl*, as a significant time difference of more than 10 hours between the onset *Pt-Dl* transcription at late stage 4 and initiation of *Pt-cad* transcription at mid stage 6 has been observed (Oda et al., 2007). To better understand the regulation and role of *Pt-cad* therefore, I first aimed to characterise the expression of *Pt-cad* in greater detail throughout the stages of posterior segment formation.
4.1 Expression of the caudal ortholog in Parasteatoda

From mid stage 6 on *Pt-cad* is expressed in a circular domain in the SAZ (see fig. 18 A), from which it then clears centrally (see fig. 18 B). Subsequently, *Pt-cad* is expressed in a broad anterior crescent shaped domain and a posterior circular domain (see fig. 18 C). At late stage 7, *Pt-cad* expression appears in the mesoderm of the prosoma in a 1 cell wide stripe and is also strongly expressed in the anterior portion of the O1 segment and throughout the SAZ (see fig. 18 D). The stripe of expression in the prosoma broadens in width to up to 3 cells, whereas expression in the forming O1 segment fades (see fig. 18 E). At this stage, *Pt-cad* is expressed throughout the entire SAZ with stronger expression at the anterior and in a posterior domain (see fig. 18 E). At stage 8.2, *Pt-cad* is expressed strongly in the SAZ and shows faint expression in O2 and strong expression in a 4-5 cells wide stripe in the presumptive L4 segment (see fig. 18 F).
Fig. 18 | *Pt-cad* wildtype expression during stages 6-8.1. Whole mount embryos in a ventral view of stage 6 embryos (A, B) and opisthosomal germ band (C-F), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-cad* expression starts in a circular domain in the centre of the germ disc at stage 6 (A) and subsequently clears from the centre slightly later (B). At mid stage 7, two distinct expression domains within the SAZ can be found; a circular one in at the posterior and a crescent shaped domain at the anterior portion of the SAZ (C). Further, at late stage 7, *Pt-cad* is expressed strongly in the SAZ, the anterior portion of the O1 and in a stripe (black arrow) in the mesoderm of the prosoma (D). *Pt-cad* is strongly expressed in the posterior and a stripe in the anterior portion the SAZ, the anterior portion of O1 and the stripe in the prosoma increases in width to up to 3 cells (black arrow) (E). The prosomal domain becomes broader with up to 5 cells in width (black arrow), the expression in O1 has disappeared, strong expression has developed in the newly formed O2 segment and *Pt-cad* is still expressed in the SAZ (F).
4.2 *Pt-cad* is not required for dynamic *Pt-Dl* expression in the posterior

Previous studies have investigated *Pt-Delta* and *Pt-cad* expression in relation to each other and also studied the effect of *Pt-Delta* on *Pt-cad* in the posterior (Oda et al., 2007). Based on its dynamic expression throughout posterior development, *Pt-cad* represents a potential candidate gene to feed back to Delta-Notch signaling pathway (Oda et al., 2007) to generate repetitive gene expression associated with the formation of each new segment.

Therefore the effect of *Pt-cad* embryonic RNAi knockdown on *Pt-Delta* expression was analyzed at stage 7, when *Pt-Dl* and *Pt-cad* are expressed in distinct domains but do exhibit overlap in the SAZ (chevrons, fig. 19 A).

In the embryonic RNAi experiment, no effect on the expression level of *Pt-Dl* could be observed in *Pt-cad* knockdown clones in

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**Fig. 19 | The effect of *Pt-cad* on *Pt-Dl***

Whole mount embryos in a ventral view of stage 6 embryos (A-B). Flat mount embryos with the anterior to the left and the posterior to the right. In wild-type embryos at stage 7 *Pt-Dl* (blue) and *Pt-cad* (orange) are expressed in distinct but also overlapping domains (indicated by chevrons and arrowheads) in the SAZ (A). In *Pt-cad* eRNAi knockdown cell clones (red), *Pt-Dl* expression (blue) appears unaffected.
the SAZ that overlapped with cells that express both Pt-Dl and Pt-cad in wild-type embryos (see fig. 19 B). This suggests that Pt-cad is not involved in the regulation of Pt-Dl during posterior development in Parasteatoda.

4.3 Characterizing the expression of pair-rule orthologs throughout segmentation in Parasteatoda

Comparative studies have shown that components of the Drosophila segmentation cascade are involved in segmentation in both long and short germ arthropods (Choe et al., 2006; Copf et al., 2004; Damen et al., 2000; Dearden et al., 2002; Liu and Kaufman, 2005; Mito et al., 2005; Shinmyo et al., 2005). For example, the pair-rule orthologs in the spider Cupiennius have dynamic expression in the SAZ and are subsequently expressed in posterior segments (Damen et al., 2005).

In order to identify potential downstream targets of Delta-Notch and Wnt signaling during Parasteatoda segmentation, expression of the pair-rule gene orthologs even-skipped (Pt-eve), runt (Pt-run-1), odd-skipped (Pt-odd-1), odd-paired (Pt-opa) and sloppy-paired (Pt-slp) were characterized throughout posterior development.

4.3.1 Structure and expression of the Parasteatoda even-skipped ortholog

A single even-skipped ortholog (Pt-eve>aug3.g17585.t1, Scaffold2587:24766..40760, +strand) was identified in the Parasteatoda
Results

One *Pt-eve* transcript is transcribed from the + strand and the coding region is 884 bp.

![Phylogenetic analysis of the *Drosophila even-skipped* orthologs.](image)

**Figure 20**| Phylogenetic analysis of the *Drosophila even-skipped* orthologs. The gene trees were built by maximum likelihood analysis in PhyML (Guindon and Gascuel, 2003); branch support values (approximate Likelihood-Ratio Test) are indicated in red. The scale bar at the bottom left indicates the branch length, which is proportional to the number of substitutions per site. *Schistocerca americana* (Sa), *Drosophila melanogaster* (Dm), *Gryllus bimaculatus* (Gb), *Tribolium castanæum* (Tc), *Apis mellifera* (Am), *Nasonia vitripennis* (Nv), *Euperipatoides kanangrensis* (Ek), *Parasteatoda tepidariorum* (Pt), *Cupiennius salei* (Cs), *Platyneris dumerili* (Pd).

The Augustus annotation for the *Parasteatoda even-skipped* ortholog predicts 3 exons with sizes of 178 bp (exon1), 182 bp (exon2) and 438 bp (exon3). Furthermore, the 2 annotated introns exhibit a large difference in size (intron1 = 12210 bp, intron2 = 2908 bp). Analysis of the predicted Pt-Eve protein (255 aa) identified a DNA-binding homeodomain (51 aa), spanning exons 2 and 3 (see fig. 21 A). The homeodomain alignment using BLASTp revealed high conservation in comparison with other arthropods (see fig. 21 B).
In situ hybridisation showed that Pt-eve exhibits dynamic expression in the SAZ that resolves into stripes of expression in nascent segments over the course of posterior development (see fig 22 A-H). Pt-eve is first expressed in a small circular expression domain of approximately 15 cells in the SAZ at stage 6, during the transition from the germ discs to the germ band stage (data not shown). This expression domain then increases in diameter (fig. 22 A), but concomitantly the centre clears (fig. 22 B) to form a transient ring of expression (fig. 22 C). This ring shaped expression domain is broken by the loss of Pt-eve expression in the most posterior cells to form a stripe of expression of approximately 3 cells in width in the nascent O1 segment during stage 7 (fig. 22 D). At this stage, expression of Pt-eve is again observed in a circular domain in the most posterior cells of the SAZ (fig. 22 D), which again clears centrally (fig. 22 E) and then breaks to form a second stripe in the presumptive O2 segment, as expression begins to narrow and fade in the older O1 stripe of expression (fig. 22 F). Subsequently, Pt-eve expression undergoes similar dynamic cycles
of strong expression in the SAZ followed by clearance of expression from this region and expression in the forming segments. As *Pt-eve* stripes form in nascent segments, the expression in the older, more anterior, segments fades. Therefore, during formation of O3 (fig. 22 G) and the remaining posterior segments, *Pt-eve* expression is only observed in the two or rarely the three most posterior (and thus youngest) segments as well as dynamically in the SAZ. Expression is also seen in the developing nervous system in older prosomal and opisthosomal segments (fig. 22 H).

**Figure 22 | Pt-eve exhibits dynamic expression in the SAZ and in nascent segments.** Whole mount embryos in a ventral view of stage 6 embryos (A-C) and opisthosomal germ band (D-H), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. (A) *Pt-eve* is expressed in a circular domain in the SAZ at mid stage 6 and clears centrally (black arrow) at late stage 6 (B). The expression in the posterior portion of the SAZ clears entirely and *Pt-eve* is expressed in a crescent shaped domain at the anterior of the SAZ (C). During mid stage 7 *Pt-eve* expression returns strongly in the SAZ and continues to be expressed in the forming O1 segment (D). Another cycle of clearance from the SAZ can be observed during mid stage 7 (E), followed by emerging strong expression in the nascent O2, whereby expression in the posterior portion of O1 narrows (F). At stage 8.1 strong expression in the SAZ and the newly formed O3 and O2 segment can be observed (G). Later, *Pt-eve* expression can be found in the developing nervous system and mostly the anterior portion of the SAZ (H).
4.3.2 Expression of the *Parasteatoda runt-1* ortholog

In the *Parasteatoda* genome 2 runt paralogs (*Pt-run-1>* aug3.g2762.t1, Scaffold 413:813549..851485; *Pt-run-2>* aug3.g6543.t1, Scaffold 772:494170..494699) were identified, however only one paralog exhibits segmental expression (Evelyn Schwager, personal communication), which suggests an involvement in posterior development and will be referred to as *Pt-run-1* (see fig. 23).

The *Pt-run-1* transcript is transcribed from the + strand and the coding region is 1.5 kb in length. The Augustus annotation for the *Parasteatoda run-1* ortholog predicts 5 exons and 4 introns, which exhibit a significant difference in size (intron1 = 10140 bp, intron2 = 22390 bp, intron3 = 2739 bp, intron4 = 104 bp).

*Pt-run* expression commences in a circular domain in the forming SAZ (Fig. 24 A). This expression domain clears from the centre and forms a ring shaped expression domain at the anterior of the SAZ (see fig. 24 B). *Pt-run* expression then resolves into a stripe in the future O1 segment, while a new cycle of

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**Figure 23** | Phylogenetic analysis of the *Drosophila runt* orthologs. The gene trees were built by maximum likelihood analysis in PhyML (Guindon and Gascuel, 2003); branch support values (approximate Likelihood-Ratio Test) are indicated in red. The scale bar at the bottom left indicates the branch length, which is proportional to the number of substitutions per site. *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), *Apis florea* (Af), *Bombyx mori* (Bm), *Parasteatoda tepidariorum* (Pt), *Cupiennius salei* (Cs), *Limulus polyphemus* (Lp), *Tetranychus urticae* (Tu).
expression is observed in the posterior of the SAZ (see fig. 24 C). Low levels of *Pt-run-1* expression at the anterior of O1 and a strong stripe expression domain at the anterior of the SAZ can be found at stage 8.1 (see fig. 24). A stripe in the nascent O2 segment forms and expression again clears from the SAZ (see fig. 24 E). While segments are added from the posterior, *Pt-run* expression fades from the O1 segment, but is still expressed in O2 and appears again as a circular domain in the SAZ area (see fig. 24 F).

**Figure 24 | Pt-run-1 wild-type expression.** Whole mount embryos in a ventral view of stage 6 embryos (A-C) and opisthosomal germ band (D-F), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-run-1* is expressed in a circular domain at stage 6 (A), clears from the posterior entirely and refines to a crescent shaped domain at the anterior of the SAZ (B). The expression in the anterior SAZ becomes stronger and a circular domain in the posterior SAZ arises at early stage 7 (C). At a slightly later stage, faint expression arises in the forming O1 segment and refines to a stripe in the anterior SAZ (D). *Pt-run-1* is expressed strongly in O1 and the anterior of the SAZ, but clears from the posterior at early stage 8.1 (E). Expression fades from O1, is expressed in the anterior portion of O2 and in a stripe domain at the anterior and a small patch at the posterior of the SAZ (F).
Expression of *Pt-eve* and *Pt-run* appear in similar domains over the course of posterior development and in order to study their regulatory interactions, I investigated their expression in relation to each other with double in situ hybridisation. *Pt-run-1* expression commences during stage 6 (see fig. 25 A), at approximately the same time that *Pt-eve* can be first detected (see fig. 25 A). Furthermore, *Pt-run-1* and *Pt-eve* expression partially overlap in anterior and posterior SAZ cells at the stages assayed (see fig. 25 A-C). However, *Pt-eve* is expressed approximately 3 cell rows anterior to *Pt-run-1* in forming segments (see fig. 25 A-C).

![Figure 25](image)

**Figure 25 | Double in situ hybridisation of *Pt-run-1* and *Pt-eve* at stages of posterior development.** Flat mount embryos in a ventral view of a stage 6 embryo (A) and opisthosomal germ band (B-C), respectively. In all panels anterior is to the left. *Pt-run-1* and *Pt-eve* expression largely overlap in the SAZ (arrow in A). Expression of *Pt-eve* is 2-3 cell rows anterior of *Pt-run-1* in forming stripes (arrow in B, C).

### 4.3.3 Expression of the *Parasteatoda* odd-skipped ortholog

In the *Parasteatoda* genome 2 odd paralogs (*Pl-odd-1* > aug3.g10084.t1, Scaffold 1114:68730..184114; *Pl-odd-2* > Locus 17047) could be identified, however only one paralog exhibits opisthosomal expression (Natascha Turetzek, personal communication) that might indicate that this gene is involved
in segmentation (see fig. 26). This gene will be referred to as *Pt-odd-1*. The *Pt-odd-1* transcript is transcribed from the - strand and the coding region is 1.3 kb in length. The Augustus annotation for the *Parasteatoda run-1* ortholog predicts 4 exons.

**Figure 26 | Phylogenetic analysis of the *Drosophila odd-skipped* orthologs.** The gene trees were built by maximum likelihood analysis in PhyML (Guindon and Gascuel, 2003); branch support values (approximate Likelihood-Ratio Test) are indicated in red. The scale bar at the bottom left indicates the branch length, which is proportional to the number of substitutions per site. *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), *Bombyx mori* (Bm), *Parasteatoda tepidariorum* (Pt), *Cupiennius salei* (Cs), *Euperipatoides kanangrensis* (Ek).

*Pt-odd-1* expression comes on in the anterior at stage 8.2 in the developing stomodaeum region (data not shown), which persists throughout the stages 8.1-12 (see fig. 27 A-C). At stage 9.2, in the mesoderm of all developing prosomal appendages, a ring-shaped domain of expression can be observed at the base, very faintly within the forming appendages (probably corresponding with segmental borders) and a circular domain at the tip of each appendage (see fig. 27 A). In the opisthosoma, *Pt-odd-1* is expressed at the anterior of O2 and O3 (see fig. 27 A). At stage 10, in the anterior two domains appear in the labrum and expression in the stomodaeum is still visible (see fig. 27 B). The *Pt-odd-1* expression domains at the tip of the developing limbs become stronger.
(chevron in fig. 27 B) and in the opisthosoma, expression appears in O3-O5 (see fig. 27 B) at stage 10. At stage 11, Pt-odd-1 expression in the anterior is restricted to the stomodaeum (see fig. 27 C). Further, the number of circular Pt-odd-1 expression domains increases (probably corresponding with the increasing development of segmental borders) (see fig. 27 C). Pt-odd-1 is also expressed in a stripe in the posterior at stage 11 (asterisk in fig. 27 C). Expression of Pt-odd-1 first emerges in the developing head and prosoma and appears in the opisthosoma only during later stages of development. Thus, the Pt-odd-1 expression pattern suggests that this gene does not have a crucial function in opisthosomal segment formation of Parasteatoda, but might be involved in the development of the walking legs, parts of the head and aspects of opisthosomal appendages.
Figure 27 | Pt-odd-1 wildtype expression. Whole mount embryos in a ventral view of the opisthosomal germ band (A-C). All panels show the same embryo respectively in an anterior (left), a prosomal (2nd view), an opisthosomal (3rd) and a side view (4th view). In all panels anterior is to the left and embryos are counterstained with DAPI. Expression in the stomodaeeum of all observed stages is marked with an arrow (A-C). At stage 9.2, a ring-shaped expression domain can be found at the base of the forming appendages (A). There is also expression at the anterior of the O2 and O3 segments (A). At stage 10, probably in accordance with the addition of segments, more rings of expression appear in the appendage mesoderm and the expression at the tip of the appendages becomes stronger (B). With further development, the ring-shaped expression domains in the mesoderm of the appendages increase in number, as well as within the opisthosomal segments (C).
4.3.4 Expression of the Parasteatoda opa-paired ortholog

In the Parasteatoda genome one opa ortholog \((Pt\text{-}opa>aug3.g12202\text{, Scaffold 1447:114106..154022})\) was identified (see fig. 28). \(Pt\text{-}opa\) is transcribed from the + strand and the coding region is 1.3 kb in length.

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**Figure 28** | Phylogenetic analysis of the Drosophila odd-paired orthologs. The gene trees were built by maximum likelihood analysis in PhyML (Guindon and Gascuel, 2003); branch support values (approximate Likelihood-Ratio Test) are indicated in red. The scale bar at the bottom left indicates the branch length, which is proportional to the number of substitutions per site. *Nasonia vitripennis* (Nv), *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), *Glomeris marginata* (Gm), *Parasteatoda tepidariorum* (Pt), *Cupiennius salei* (Cs), *Euperipatoides kanangrensis* (Ek).

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\(Pt\text{-}opa\) expression appears at stage 8 in the developing head and the prosoma. At stage 9.1, \(Pt\text{-}opa\) is expressed in a distinct domain in the PcL (see fig. 29 A), which then splits into an anterior (arrow) and a lateral (arrow head) domain. The superficial ectodermal layer of L1-L4 does not show any signal, but faint expression can be observed in the mesoderm (see fig. 29 B). Interestingly, expression in L2 is stronger compared to expression in the other leg bearing segments (see fig. 29 B). Also at stage 10, a specific domain in the mesoderm of the O2 segment can be observed (see fig. 29 B). In the developing head, expression in the PcL persists (black arrow, arrowhead) and an additional domain appears at the labrum. The mesodermal expression in the limbs
persists and the expression in the appendage of the opisthosomal appendage O2 becomes stronger (see fig. 29 C). Inferred from the expression analysis, Pt-opa might be involved in brain development, limb formation and aspects of book lung growth.

Figure 29 | Pt-opa wildtype expression. Whole mount embryos in a ventral view of the opisthosomal germ band (A-C). Panels B, C show the same embryo respectively in an anterior (left), a prosomal (2nd view), opisthosomal (3rd) and a side view (4th view in C). In all panels anterior is to the left and embryos are counterstained with DAPI. Pt-opa expression commences at stage 9.1 in a distinct domain in the PcL (black arrow, A). Further, expression in the PcL splits into two different domains at the anterior (arrow) and a lateral (arrow head) domain (B). At stage 10, faint expression in the developing limb mesoderm can be observed, whereby expression in L2 is stronger compared to the leg bearing segments and there is also a specific domain in the O2 segment (B). At stage 11, the expression in the PcL still persists (black arrow, arrow head) and in addition, an expression domain at the labrum (asterisk) appears (C). The mesoderm expression in the walking legs is unchanged and the expression in the opisthosoma (book lung opening at the posterior of O2) becomes stronger (chevron) (C). The lateral view shows that in addition to the limb mesoderm, there is also expression at the base of each prosomal segment (C).
4.3.5 Expression of the *Parasteatoda sloppy-paired* ortholog

In the *Parasteatoda* genome a single sloppy-paired ortholog (*Pt-slp* >aug3.g19520, Scaffold 3303:1..36954) could be identified (see fig. 30). The *Pt-slp* transcript is transcribed from the - strand and the coding region is 1.3 kb in length. The Augustus annotation for the *Parasteatoda opa* ortholog predicts 4 exons and 4 annotated introns, which exhibit a significant difference in size (intron1= 4276 bp, intron2=15962 bp, intron3=12316 bp, intron4=3105 bp).

![Phylogenetic analysis of the Drosophila sloppy-paired orthologs.](image)

Expression of *Pt-slp* commences at stage 8.1 in the head lobes in a triangular domain and in the central portion of the prosomal segments L1-L4 (see fig. 31A). The triangular domains of the developing head become broader at stage 9.1 and two circular domains appear at the labrum and undefined expression around the stomodaeum (see fig. 31B). The expression in the prosomal and opisthosomal segments becomes stronger and is restricted to the anterior
portion of each segment, excluding the midline (see fig. 31 B). *Pt-slp* expression in the head lobes expands antero-laterally and faint expression arises in the nervous system (see fig. 31 C). The segmental expression becomes more U-shaped in the Pp, Ch, L1-4 and opisthosomal segments (see fig. 31 C). *Pt-slp* expression is not expressed in the SAZ at any of the observed stages, only in the newly formed opisthosomal segments. Due to the expression of *Pt-slp* in the head and the ventral ectoderm, I suggest that *Pt-slp* might be involved in the development of the brain and the central nervous system.
Figure 31 | *Pt-slp* wildtype expression. Whole mount embryos in a ventral view of the opisthosomal germ band (A-C). All panels show the same embryo respectively in an anterior (left), a prosomal (2nd view), opisthosomal (3rd) and a side view (4th view in C). In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-slp* expression arises in the head lobes (arrow head) and the central part of the prosomal segments (A). Expression in the head lobe exhibits a triangular shape and becomes stronger (arrow head) (B). There is also *Pt-slp* expression around the stomodaeum (arrow) and at the labrum (asterisk) at stage 9.1 (B). Within the prosomal segments and the opisthosomal segments O1-O5, expression can be observed at the anterior of each segment and in a circular lateral domain (B). At stage 10, the anterior expression domain becomes larger at the anterior/lateral part of the head lobes (arrow head) and expression in the developing nervous system appears (arrow; C). Strong expression can still be observed in the anterior of the prosomal segments, whereby the lateral domain is fused with the stripe domain at this stage (C).
4.4 Discussion

4.4.1 Expression and role of the caudal ortholog in Parasteatoda

I have characterised the expression of *Pt-cad* in greater detail compared to previous work (McGregor et al., 2008b; Oda et al., 2007). This shows that it comes on at a similar stage to *Pt-Dl* in the germ disc and subsequently exhibits dynamic expression in the SAZ and is expressed in new segments (see fig.15 and fig. 18). I also tested if *Pt-cad* has an effect on the dynamics of *Pt-Dl* expression and found that *Pt-cad* is not involved in regulating *Pt-Dl* (Schonauer et al., 2016). However, since *Pt-Dl* is required for *Pt-cad* expression (Oda et al., 2007), my results confirm again that *Pt-cad* must be downstream of Delta-Notch signaling in the spider, whilst it is still unclear if *Pt-cad* is a direct target of *Pt-Dl* signaling or if there are intermediate factors (Oda et al., 2007; Schonauer et al., 2016).

In *Strigamia*, caudal (*Sm-cad*) expression precedes morphological segmentation with uniform expression in undifferentiated cells of the blastodisc (Chipman et al., 2004). Throughout posterior development, *Sm-cad* expression is also continuously maintained in the posterior disc and only contracts around the proctodeum upon segment formation (Chipman et al., 2004), in contrast to *Parasteatoda*, where *Pt-cad* expression in the posterior SAZ is more dynamic, alternating between broad expression and clearing from this area (Schonauer et al., 2016). In association with the subsequent elongation of the germ band, *Sm-cad* expression resolves into stripes, reminiscent of *Pt-cad* expression in the spider (Chipman et al., 2004; Schonauer et al., 2016). However, *Sm-cad* expression appears in a double-segmental pattern, in contrast to the single
segmental expression in *Parasteatoda* (Chipman et al., 2004; Schonauer et al., 2016).

The expression profile of *cad* in *Periplaneta* also differs from the spider. *Pa-cad* is expressed broadly in the GZ and establishes a boundary between the undifferentiated GZ and differentiated cells of the forming segments. However, *Pa-cad* is also regulated by Delta-Notch and Wnt signaling in this cockroach (Chesebro et al., 2013) although in contrast to the spider, *Pa-cad* appears to repress *Pa-DI* (Chesebro et al., 2013). This suggests that while arthropods like *Parasteatoda* and *Periplaneta*, that use similar components including Delta-Notch signaling in GRNs for posterior development, there are differences in their interactions that have evolved since the common ancestor. Indeed, in *Tribolium*, it appears that Delta-Notch signaling may not be employed in posterior segmentation (Aranda et al., 2008), and *Tc-cad* is a maternally deposited morphogen in this beetle which regulates posterior development through control of the spatio-temporal expression of pair-rule genes (Copf et al., 2004; El-Sherif et al., 2014).

### 4.4.2 Expression analysis of pair-rule gene orthologs in *Parasteatoda*

In this chapter I also report that I found that *Pt-eve* and *Pt-run-1* are expressed in the SAZ and in nascent segments (see fig. 25). The expression domains of both genes predominantly overlap, with *Pt-eve* 2-3 cell rows anterior of *Pt-run-1* at the stages investigated (see fig. 25). As expected, the expression of these genes is similar between *Parasteatoda* and *Cupiennius* (Damen et al., 2000; Schonauer et al., 2016).
Interestingly, the relative expression of pair-rule ortholog genes during segmentation has diverged among arthropods, as the expression of eve and run do not overlap in Strigamia and Drosophila, but they do overlap in Parasteatoda and Glomeris (Frasch et al., 1987; Green and Akam, 2013; Janssen et al., 2011; Schonauer et al., 2016).

In Strigamia, three even-skipped paralogs have been identified and they differ in expression: Sm-eve1 is expressed in a double segmental pattern in the posterior disc and resolves into a single segmental expression in the germ band. Sm-eve2 is only expressed in the posterior disc and does not resolve into a stripe pattern in the germ band and Sm-eve3 is only expressed in the transition zone, where the germ band arises. However, all three Sm-eve paralogs are in phase with each other and hence all three of them are out of phase with Sm-run expression (Green and Akam, 2013).

In the spider, the function of Pt-eve and Pt-run-1 appears to be restricted to the posterior and no expression was detected in anterior structures like the prosoma or head, unlike in other arthropods (Brena and Akam, 2013; Brown et al., 1997; Frasch et al., 1987; Janssen et al., 2011; Schonauer et al., 2016). This is further evidence that patterning of the prosoma and the opisthosoma is regulated differently in the spider (Damen et al., 2005; Damen et al., 2000; Pechmann et al., 2011; Pechmann et al., 2009; Schwager et al., 2009).

It has been first established in Drosophila that the pair-rule genes can be distinguished by their regulation and function in primary and secondary pair rule genes. In the fruit fly eve acts as a primary and run as a secondary pair-rule gene (Ingham, 1988), while it has been found in Tribolium that eve and run both
act as primary pair rule genes (Choe et al., 2006). Also in the centipede, *Sm-eve1* and *Sm-run* were classified as primary pair-rule genes, due to their early onset and the double-segmental expression (Green and Akam, 2013). While not all potential regulatory input factors are known, I propose that *Pt-eve* and *Pt-run* act on the same hierarchical level and although they are expressed with single segmental periodicity they potentially act upstream of other pair-rule gene orthologues and thus represent primary-pair rule genes in the spider (Damen, 2007).

Therefore, for further analysis, I suggest testing the interactions between *Pt-eve* and *Pt-run* and other pair-rule genes like *Pt-slp* and *Pt-odd-1*, which show later segmental expression that probably overlaps with either *Pt-cad* or *Pt-eve* and *Pt-run-1*. If *Pt-slp* and *Pt-odd* act as secondary pair-rule genes, I would expect to see an effect in *Pt-eve* and *Pt-run-1* knockdown clones and no effect in *Pt-cad* clones.

In *Parasteatoda*, *Pt-odd* skipped is expressed in the developing walking legs, the developing head and opisthosomal segments at later stages (see fig. 27). One *odd-skipped* gene has been described in *Drosophila*, which is responsible for the specification of the anterior regions of the segments through interactions with the primary pair-rule genes *even-skipped* and *fushi-tarazu* (Coulter and Wieschaus, 1988). In *Tribolium odd-skipped* is expressed in a double-segmental pattern and has been found to repress *Tc-eve* expression in the pair-rule gene circuit (Choe et al., 2006). In *Cupiennius* expression of the *odd-skipped-related* gene was only detected in the anterior portion of the SAZ and
exhibits a transient pattern which disappears as soon as the segment forms (Damen et al., 2005).

In *Parasteatoda*, *Pt-opa* exhibits faint expression in the developing walking legs, the head and in opisthosomal appendages (see fig. 29). Whilst *odd-paired* expression is initially ubiquitous in *Drosophila*, it does resolve into segmental expression at later stages (Benedyk et al., 1994). In *Cupiennius Cs-opa* is expressed in two stripes in the SAZ and in broad single-segmental stripes in the segments (Damen et al., 2005). This is in contrast to *Parasteatoda*, where *Pt-opa* expression is absent from the opisthosoma at stages of SAZ formation and segmentation. Only at stage 12 a circular domain appears in the O2 segment, which might be associated with the development of appendages arising from this area (see fig. 29 C, chevron). This suggests that *Pt-opa* is not involved in the SAZ and segment formation and the difference between *Pt-opa* and *Cs-opa* expression suggests that they might not be homologs.

*Pt-slp* expression in *Parasteatoda* displays segmental expression in prosomal and opisthosomal segments and is expressed in the developing head (see fig. 31). In *Drosophila*, two *sloppy paired* paralogs have been identified (*slp1, slp2*), with almost identical expression patterns (Grossniklaus et al., 1992). *Slp1* and *slp2* are secondary pair-rule genes, maintaining segment boundaries downstream of *eve* and also exhibit redundant function in neurodevelopment (Cadigan et al., 1994; Grossniklaus et al., 1992). *Tribolium sloppy-paired* expression commences in anterior segments and resolves into a double-
segmental pattern segmentation (Choe and Brown, 2007). The functional analysis for Tc-slp revealed a role in gnathal segment formation, development of even-numbered segments and maintenance of odd-numbered segments of the trunk (Choe and Brown, 2007).

In Cupiennius, Cs-slp expression is segmental and restricted to the ventral portion of the segments, similar to what I found in early stages of Parasteatoda development (see fig.10 A, stage 8.1) (Damen et al., 2005). Pt-slp expression also suggests that this gene is involved in nervous system development (fig.31 B,C), like in Drosophila. However, my results suggest that Pt-slp is not involved in the formation for the SAZ, but potentially in maintaining the segmental borders because it is expressed in fully formed segments.
5 Results Chapter 3:
    Characterising the GRN underlying posterior segmentation, focusing on the regulatory interactions involving the pair-rule ortholog even-skipped in Parasteatoda

5.1 The pair-rule genes *Pt-eve* and *Pt-run-1* are regulated by Wnt and Delta-Notch signaling in the posterior

The dynamic expression of *Pt-eve* and *Pt-run-1* in the SAZ and in the forming opisthosomal segments suggests an involvement of these genes in SAZ formation and segmentation in *Parasteatoda*. Furthermore, the expression of *Pt-Wnt8* and *Pt-Delta* (McGregor et al., 2008b; Oda et al., 2007) precede the onset of *Pt-eve* and *Pt-run-1*, suggesting that the pair-rule gene orthologs act downstream, similar to the effect on *Pt-cad*. Hence, the effect of *Pt-Wnt8* and *Pt-Delta*, on *Pt-eve* and *Pt-run-1* were tested.

Knockdown of *Pt-Delta* with pRNAi caused the complete loss of the expression of both *Pt-eve* and *Pt-run-1* in the SAZ (see figs. 32 B and 31 B). *Pt-eve* and *Pt-run-1* expression was also greatly reduced after pRNAi knockdown of *Pt-Wnt8* RNAi with only a few remaining cells expressing each gene (see fig. 32 C and fig. 33 C). Previous studies of *Pt-Wnt8* RNAi phenotype embryos (McGregor et al., 2008b) suggest that the *Pt-Wnt8* knockdown effect is not complete in all embryos which may explain why a few cells still express *Pt-eve* and *Pt-run-1* expression.
Results

Figure 32 | *Pt-eve* expression in *Pt-Dl* and *Pt-Wnt8* RNAi embryos. Whole mount embryos in a ventral view of stage 6 embryos (A-C). In all panels anterior is to the left and embryos are counterstained with DAPI. Panels A’-C’ show the DAPI staining of the respective bright field/DAPI overlay images A-C. *Pt-eve* wild-type expression in the centre of the germ disc at the stage analysed in the RNAi embryos (A). In *Pt-Dl* pRNAi embryos, *Pt-eve* expression is no longer detectable in the SAZ (B). In *Pt-Wnt8* pRNAi embryos expression of *Pt-eve* is reduced to only a few cells (C). The dashed circle in B indicates the SAZ A.
Intriguingly, the effects of Pt-Wnt8 and Pt-Delta RNAi on the expression of Pt-eve and Pt-run-1 are strongly reminiscent of the effect of knockdown of these genes on Pt-cad expression (McGregor et al., 2008b; Oda et al., 2007). Therefore, I next investigated if Pt-cad is also involved in the regulation of Pt-eve and Pt-run-1.
5.2 The effect of Pt-cad on Pt-eve

In order to investigate the possibility that Pt-cad could regulate Pt-eve, I first, analysed the expression of these two genes in relation to each other during posterior development.

Pt-cad and Pt-eve expression are initially detected at mid stage 6 in a small domain in the centre of the germ disc (see fig. 34 A). Pt-eve clears from the central part, whereby Pt-cad expression persists in this domain (see fig. 34 A'). Subsequently, Pt-eve and Pt-cad expression expands into an overlapping crescent shaped domain at the anterior of the SAZ, but Pt-cad expression then persists in the more anterior cells from which expression of Pt-eve has cleared (see fig. 34 B). At stage 7, both Pt-eve and Pt-cad are expressed in a partially overlapping stripe in the nascent O1 segment: Pt-eve is expressed in the anterior-most row of cells, followed by two rows of cells with overlapping expression and Pt-cad is expressed alone in approximately two rows of the most posterior cells of the stripe (see fig. 34 C). At this stage a new domain of overlapping expression of Pt-eve and Pt-cad can also be observed in posterior SAZ cells (see fig. 34 C). The two genes continue to be expressed in a similar fashion during the subsequent addition of segments (see fig. 34 D, E). Thus the relative expression patterns of Pt-cad and Pt-eve suggest that there might be a regulatory interaction between these two genes, due to a significant degree of overlap.
Despite several attempts previously, the knockdown of *Pt-cad* with parental RNAi did not result in any obvious phenotype (McGregor, personal communication). However, I showed that I could knockdown *Pt-cad* expression in clones by applying eRNAi (Kanayama et al., 2011; Kanayama et al., 2010) (also see chapter 2.4.3.) using two dsRNAs, corresponding to two non-overlapping fragments of the *Pt-cad* coding region (*Pt-cad* fragment 1 n=16, *Pt-cad* fragment 2 n=11) (data not shown).

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**Figure 34 | Pt-eve and Pt-cad wild-type expression.** Whole mount embryos in a ventral view of stage 6 embryos (A-C) and opisthosomal germ band (C-E), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-cad* (orange) and *Pt-eve* (blue) are initially co-expressed in about 15 cells in the SAZ (A). However, *Pt-cad* expression remains in cells, where *Pt-eve* has cleared again (black arrows) (A'). Expression of both genes then clears from the posterior and *Pt-cad* and *Pt-eve* are expressed in an overlapping crescent shaped domain, where *Pt-eve* is expressed more anteriorly (B). Subsequently, *Pt-eve* and *P-cad* are both again expressed in the posterior SAZ cells (C) with successive clearing, and in one (D) or two (E) of the youngest segments. The *Pt-cad* expression is broader than that of *Pt-eve* and persists for longer in the SAZ (D-F).
The effect of *Pt-cad* eRNAi on *Pt-eve* expression during different stages of posterior development was then investigated (n=16) (see fig. 35 B, D). At stage 6, *Pt-eve* expression was lost or strongly reduced from cells subject to *Pt-cad* knockdown (see fig. 35 B). Similarly, *Pt-cad* eRNAi also results in reduced *Pt-eve* expression within the nascent segment and the SAZ at stage 7 (see fig. 35 D). These results suggest that *Pt-cad* is required for *Pt-eve* expression in *Parasteatoda* and confirms the hypothesis drawn from the previous results that Wnt and Delta-Notch signaling act at least in part via *Pt-cad* to regulate segmentation genes like *Pt-eve*.

**Figure 35** | The effect of *Pt-cad* on *Pt-eve* expression. Whole mount embryos in a ventral view of stage 6 embryos (A-B) and opisthosomal germ band (C-D), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. The effect of *Pt-cad* RNAi on *Pt-eve* in the SAZ and the nascent segments was observed in 16 injected embryos in total.
5.3 The effect of Pt-cad on Pt-run-1

Pt-cad and Pt-run-1 are both expressed in the SAZ, with alternating phases of clearing and strong expression, and in stripes in the forming segments (see fig. 18 A-F and fig. 24 A-F). The expression of Pt-run-1 in relation to Pt-cad is reminiscent of to the relative expression of Pt-eve to Pt-cad (see fig. 34 A-E), whereby Pt-eve is 2-3 cell rows anterior of Pt-run-1.

Since the effect of pRNAi against Delta/Notch and Wnt8 on Pt-run-1 expression (see fig. 36 B, C) is reminiscent of the result on Pt-eve expression, it has been suggested that this may also be indirect through the loss of Pt-cad. Therefore, I then tested whether Pt-cad is also required for Pt-run-1 expression. I found that eRNAi against Pt-cad results in the loss of Pt-run expression in SAZ cells suggesting that Pt-cad is required for Pt-run-1 expression (1), as well as Pt-eve expression (see fig. 36 B).

Figure 36 | Pt-cad activates Pt-run-1 expression. Whole mount (A) and a flat mount (B) embryos of the opisthosoma (B). At stage 6, Pt-run-1 is expressed in a circular domain in the SAZ (C), in a similar expression domain to Pt-cad at this stage (see fig 17 A). Pt-run-1 expression is down regulated in the Pt-cad knockdown clone in the SAZ (B).
5.4 *Pt-cad* is not sufficient for the activation of *Pt-eve*

Since the above experiments show that *Pt-cad* expression necessary to activate *Pt-eve* expression, I then tested if it is sufficient. To do this I injected capped *Pt-cad-eGFP* mRNA into blastomeres at the 16-cell stage and allowed them to develop until stage 5 (i.e. before *Pt-cad* and *Pt-eve* are normally expressed). Clones of cells with nuclear GFP expression were observed (see fig. 37 B), demonstrating that *Pt-cad* was expressed and able to localise to the nuclei (n = 5). These embryos were fixed at stage 5 and an in situ hybridisation for *Pt-eve* was carried out. However, I did not observe expression of *Pt-eve* in any of these cells even after staining until background started to appear. This indicates, that while *Pt-cad* expression is required for *Pt-eve* expression, it is not sufficient in these conditions (see fig. 37 C). Indeed, since some of these cells expressing *Pt-cad*-GFP near the pole of the germ at this stage are likely to also express *Pt-Wnt8* and *Pt-Dl* (McGregor et al., 2008b; Oda et al., 2007), this implies that an additional factor or factors are required to activate *Pt-eve* (see fig. 37 A).
I then tested if \textit{Pt-eve} feeds back to regulate \textit{Pt-cad} expression. As for \textit{Pt-cad}, pRNAi knockdown for \textit{Pt-eve} did not appear to work (McGregor personal communication). Therefore, eRNAi was successfully established for \textit{Pt-eve}: the microinjection of dsRNA covering the entire \textit{Pt-eve} coding region resulted in a loss of \textit{Pt-eve} expression (n = 4). However, the knockdown of \textit{Pt-eve} using eRNAi did not appear to affect \textit{Pt-cad} expression in the SAZ and in the forming
segments unaffected (n = 16) (see fig. 38 B, D). This indicates that Pt-eve does not regulate Pt-cad expression and is thus downstream of Pt-cad in the GRN of segmentation in Parasteatoda.

Figure 38 | *Pt-eve does not have an effect on Pt-cad expression.* Whole mount embryos in a ventral view of stage 6 embryos (A-B) and opisthosomal germ band (C-D), respectively. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-cad* is expressed in a circular domain at stage 6 (A) and continues to be expressed in the posterior and an anterior SAZ domain at mid stage 7 in wild-type embryos (C). In the areas where the *Pt-eve* knockdown clone overlaps with the *Pt-cad* domain, expression is unaffected in the SAZ at mid stage (B) and also in the forming segment at mid stage 7 (D).
5.6 *Pt-eve* and *Pt-run-1* do not regulate each other

In *Tribolium*, it has been reported that a regulatory circuit directs the expression of pair-rule genes in a clock-like mechanism in order to form segments from the SAZ (Choe et al., 2006). In this model *Tc-eve* activates *Tc-run*, which further activates *Tc-odd* and which in turn represses *Tc-eve* (Choe et al., 2006). To test if a similar circuit operates in *Parasteatoda*, I investigated the regulatory interactions between *Pt-eve* and *Pt-run-1*.

To test if *Pt-eve* regulates *Pt-run* in *Parasteatoda* eRNAi knockdown of *Pt-eve* was carried out and the effect on *Pt-run-1* expression at different stages of posterior development was assayed. At all observed stages, no detectable effect of the *Pt-eve* knockdown on *Pt-run* expression could be found (n=12) (see fig. 39 B). This suggests that, in contrast to the pair-rule circuit in *Tribolium*, *Pt-eve* is not required to activate *Pt-run-1* during segment addition in *Parasteatoda*. 

Results
I then tested whether knockdown of *Pt-run-1* affected *Pt-eve* expression. I first showed that two non-overlapping fragments of the *Pt-run-1* CDS were able to knockdown *Pt-run-1* expression in the SAZ and segments (n = 13) (see fig. 40 A, B). However, injection of *Pt-run-1* dsRNA had no discernable effect on *Pt-eve* expression in the SAZ (see fig. 40 D) or in the forming segments (n = 7) (see fig. 40 C). This suggests that *Pt-run-1* does not inhibit *Pt-eve* in *Parasteatoda* and thus does not support the hypothesis of a pair-rule gene circuit regulating segmentation in the spider, at least not exactly like the model described in *Tribolium* (Choe et al., 2006).
Results

**Figure 40 | Pt-run-1 does not affect Pt-eve expression.** Flat mount embryos of the opisthosoma (A-D). In all panels anterior is to the left. Two non-overlapping fragments of the Pt-run-1 CDS, Pt-run-1 F1 (A) and Pt-run-1 F2 (B), were tested for the knockdown of Pt-run-1 expression. At mid stage 7 Pt-eve is expressed in the posterior SAZ and in anterior stripe (C), largely overlapping with Pt-run-1 expression (see fig A). Pt-eve expression appears normal in the Pt-run-1 knockdown clone in the nascent segment (C) and in the SAZ (D).
5.7 Discussion

I have found that while the knock down of either Pt-Wnt8 or Pt-Dl affects Pt-eve and Pt-run-1 expression, this is probably not a direct effect, but is mediated through Pt-cad, which I have shown is required for the expression of both these pair-rule gene orthologues (Schonauer et al., 2016). It appears that Pt-cad may not be sufficient to activate Pt-eve, however, which would suggest that other factors are required to activate Pt-eve and Pt-run-1 expression that may or may not depend on Wnt8 and Delta-Notch signaling.

Furthermore, I tested if Pt-eve expression has an effect on Pt-cad. However, no discernable effect on Pt-cad expression was observed, which suggests that Pt-eve is downstream of Delta-Notch/Wnt8/Cad in Parasteatoda. However the results of the Pt-cad overexpression experiment may have to be questioned in terms of the functionality of the tagged Pt-Cad protein: it is not clear if the Pt-Cad protein is folded correctly and hence functional, since the protein structure might be affected by the GFP tag. To follow this up, GFP could be replaced by a smaller tag (e.g. HA tag ~100bp) in order to label Pt-Cad. Furthermore, the position of the GFP tag at the C-terminus might interfere with the function of homeodomain, which is only ~ 30 aa upstream of the C-terminus. Therefore, tagging the Pt-Cad protein at the N-terminus could help prevent potential interference with folding and function of the protein in this case. Moreover, it would be desirable in terms of a control, to be able to detect the Pt-Cad protein itself, rather than just the marker, i.e. with an antibody staining, however, as far as I am aware there is no cross-reacting antibody available.
In *Tribolium*, Tc-eve is also regulated by Tc-cad (El-Sherif et al., 2014) and therefore, also considering findings in *Periplaneta* (Chesebro et al., 2013), it appears that the regulation of eve by cad may have been ancestral feature of arthropods. Furthermore in the proposed pair-rule circuit in *Tribolium*, Tc-eve activates Tc-run, which in turn activates Tc-odd (Choe et al., 2006). Tc-odd then represses Tc-eve in even-numbered parasegments and thus primary Tc-eve stripes are generated (Choe et al., 2006). Given the largely overlapping expression patterns in the SAZ and the developing segments of *Parasteatoda*, I tested if a similar circuit operated in this spider. However, the knockdown of Pt-eve left Pt-run-1 unaffected and there was also no effect on Pt-eve in Pt-run-1 knockdown clones. This suggests that the pair-rule gene orthologs examined are not connected in a *Tribolium*-like pair-rule gene circuit (Choe et al., 2006; Schonauer et al., 2016). On the contrary the effect of Pt-Dl, Pt-Wnt8 and Pt-cad RNAi knockdown on Pt-eve and Pt-run-1 suggests that pair-rule gene orthologs are instead only regulated by such upstream factors. Summarizing these results, I suggest that pair-rule gene orthologues in the spider are not regulated by a pair-rule gene circuit, but a Delta-Notch/Wnt/Cad organizer, which might be ancestral to all arthropods (Chesebro et al., 2013; Schonauer et al., 2016). Furthermore, my results suggest that the pair-rule gene circuit as established in *Tribolium* is a derived mechanism for generating a segmental pattern that may not be dependent on Delta-Notch signaling.
6 Results Chapter 4: Investigating the expression of frizzled receptor genes during spider embryogenesis

During embryonic development Wnt signaling is fundamental for cell-cell communication in multiple developmental processes like cell division, cell fate decision, cell morphology and cell movement (Logan and Nusse, 2004). In the case of the canonical pathway, secreted Wnt glycoprotein ligands bind the 7-transmembrane receptors of the Frizzled family and a lipoprotein receptor-related protein (LRP) co-receptor (arrow in Drosophila) and thereby trigger the phosphorylation of the downstream factor Dishevelled. This results in the inhibition of a multi-protein complex (including GSK3 (glycogen synthase kinase 3), APC (adenomatosis polyposis coli protein) and Axin) that normally leads to the degradation of β-catenin (Komiya and Habas, 2008), which instead now increases in concentration and enters the nucleus where it binds to LEF/TCF regulates transcription (Behrens et al., 1996) (see fig. 41).
Wnt ligands

Metazoans have 13 Wnt ligands, although deuterostomes have lost Wnt A (Kusserow et al., 2005) and protostomes have lost Wnt3 (Cho et al., 2010; Garriock et al., 2007; Janssen et al., 2010). Parasteatoda has 12 Wnt ligand genes, having lost Wnt9 and Wnt10 but containing duplicates of Wnt7 and Wnt11 (Janssen et al., 2010). Insects on the other hand have lost several Wnts and only Wnt9 and Wnt7 are found in Tribolium and Drosophila respectively (Janssen et al., 2010). However, the loss of Wnts in some insects does not seem to represent a general arthropod feature, as the crustacean Daphnia has
Results

retained 12 and the myriapod Strigamia 11 Wnt ligands (Hayden and Arthur, 2014; Janssen et al., 2010). Comparative analysis of Wnt expression and function across protostomes illustrated that many Wnt ligands are likely involved in segment formation (Hogvall et al., 2014; Janssen et al., 2010; Murat et al., 2010).

Frizzled receptors

Other functionally important components of the Wnt signaling pathway are the frizzled receptors, which consist of a conserved cysteine-rich domain (CRD), followed by a variable region (see fig. 42). The adjacent 7 trans-membrane domain transverses the plasma membrane and is followed by the N-terminal KTXXXW motif, which is part of the intracellular domain (MacDonald and He, 2012; Park et al., 1994b) (see fig. 42). The CRD has been found to be responsible for ligand recognition and the trans-membrane domain works as an anchor for the corresponding Wnt protein. The KTXXXW motif transduces the signal through phosphorylating the intracellular downstream target Dishevelled (Huang and Klein, 2004; Umbhauer et al., 2000) (see fig. 42).

Figure 42 | General structure of the frizzled receptors. The cysteine-rich domain (CRD) is located at the C-terminal end, adjacent to a variable part of the receptor. The 7 trans-membrane domain, which transverses the cell membrane, binds the Wnt ligand, whereas the KTXXXW motif at the N-terminus, activates the intracellular downstream cascade.
Four frizzled genes have been described in *Drosophila* and these receptors are involved in cell polarity and amongst other functions, regulate bristle orientation in epidermal cells (Adler, 2002; Wang et al., 1996). In *Drosophila*, Frizzled and DFrizzled-2 both act as *wingless* (*wg*) receptors, which amongst other functions, maintain *en* expression in an adjacent stripe of all developing segments (Bhanot et al., 1996; Bhanot et al., 1999; Martinez-Arias and Lawrence, 1985). In *Tribolium* there are three frizzled genes (*Tc-Fz1, Tc-Fz-2, Tc-Fz-4*) and the co-receptor *arrow*, regulating GZ maintenance, axis elongation and leg development (Beermann et al., 2011; Bolognesi et al., 2009). The knockdown of both *Tc-Fz1* and *Tc-Fz2* and *Tc-arrow*, respectively caused a reduction of the GZ and malformation of the pre-segmental region, located just anterior to the GZ. This functional analysis evidenced a crucial role for Wnt signaling in the posterior of the beetle during axis elongation and segmentation (Beermann et al., 2011).

Wnt signaling has been shown to be essential for segmentation in spiders (McGregor et al., 2008b) and four frizzled receptors have been identified in *Parasteatoda* (Janssen et al., 2015), however its unclear which Wnt ligands use which receptor for signal transduction. To gain insights into frizzled receptor evolution and to investigate the potential role of frizzled receptors in spider segmentation, I studied the expression of frizzled receptors in *Parasteatoda*. 
6.1 Analysis of the *Parasteatoda* frizzled receptors expression over the course of embryonic development

To investigate the roles of the four frizzled receptors (*Pt-fz1, Pt-fz2, Pt-fz4a, Pt-fz4b*) during embryogenesis in *Parasteatoda* in situ hybridisation was carried out for each of these genes.

6.1.1 *Pt-fz1* expression in *Parasteatoda*

*Pt-fz1* is expressed ubiquitously at low levels during stages 5-12. However, stronger and more specific expression was observed at stage 9.1 at the margin of the segmental grooves in the ventral neuroectoderm of the prosomal and the opisthosomal segments (see fig. 43).

![Figure 43 | Expression of *Pt-Fz1*.](image)

*Figure 43 | Expression of *Pt-Fz1*.* The same embryo is shown in a prosomal (left), an opisthosomal (middle) and a lateral (right) view. Anterior is to the left and the embryo is counterstained with DAPI. (A) Expression in the ventral neuroectoderm of the segmental grooves (arrows) becomes apparent at stage 9.1.
6.1.2  *Pt-fz2* expression in *Parasteatoda*

*Pt-fz2* expression commences at stage 5 in a broad ring encompassing the germ disc (see fig. 44). During stage 6, expression is restricted to an anterior stripe encompassing the germ disc (see fig. 44 A). This anterior domain broadens during stage 7 and will become the future prosoma (fig. 44 B). *Pt-fz2* is subsequently expressed in a narrow stripe along the anterior margin of the germ band and in the prosomal segments at stage 8.1 (see fig. 44 C). At this stage expression in L3 and L4 is much broader compared to L1 and L2 (see fig. 44 C). At stage 8.2, when prosomal segments become morphologically visible and the first opisthosomal segment (O1) has formed, *Pt-fz2* is expressed in the anterior portion of each segment and in the segmental groove (see fig. 44 D). At this stage the expression at the anterior margin of the germ broadens (see fig. 44 D). At stage 9.2, strong *Pt-fz2* expression in the developing head refines to the anterior portion of each precheliceral lobe (PcL) and surrounds the stomodeum (see fig. 44 E, white arrow). Strong *Pt-fz2* expression can also be observed in the ventral neuroectoderm and the dorsal periphery of each segment and in nascent opisthosomal segments (see fig. 44 E). However, no expression of *Pt-fz2* was observed in the SAZ at any stage.
Pt-fz2 wildtype expression during stages 6-9.2. Panel D and E show the same embryo, respectively in an anterior (left), prosomal (middle), and a lateral (right) (D) or an opisthosomal (right) (E) view. In all panels anterior is to the left and embryos are counterstained with DAPI. At stage 6 Pt-fz2 is expressed in a stripe in the anterior (A), which becomes wider at a slightly later stage (B). In the developing embryo, Pt-fz2 expression expands in the forming prosomal segments, whereby expression in L3 and L4 is much broader than in L1 and L2 (C). Pt-fz2 is also expressed in a thin stripe at the anterior of the germ band at stage 8.1 (C, black arrow). At stage 8.2 Pt-fz2 is expressed in the anterior portion for each segment and in the segmental groove (D). The Pt-fz2 expression domain at the anterior margin of the developing head lobe has become broader at stage 8.2 (D). At stage 9.2, Pt-fz2 expression head lobe expression refines to the anterior of each prechelicer lobe (PcL) and the future stomodaeum area (Sto, white arrow, E). Strong expression can also be found in the ventral neuroectoderm and the dorsal periphery of each segment, however the SAZ does not show Pt-fz2 expression (black arrow indicates expression in the youngest opisthosomal segment O6, E).
6.1.3 *Pt-fz4-1* expression in *Parasteatoda*

*Pt-fz4-1* expression was first detected at stage 8.2 in the segmental groove posterior of the O2 segment and in the forming O3 segment (see fig. 45 A). *Pt-fz4-1* expression was also detected later in the mesoderm of the developing limbs and in a definite domain of future neural tissue at the precheliceral lobes at stage 9.2 (see fig. 45 B). At a later stage, *Pt-fz4-1* becomes stronger and more broadly expressed in the limb mesoderm and expands also in the ventral neuroectoderm (see fig. 45 C). The expression in the head lobes continues throughout stage 9.2 (see fig. 45 C). At stage 12 *Pt-fz4-1* is strongly expressed in the limb mesoderm and in the mesoderm of the opisthosomal segments (see fig. 45 D). The faint expression in the head is restricted to the anterior border of the lobes and the labrum (L) region (see fig. 45 D; Lb, white arrow).
Figure 45 | *Pt-fz4-1* wildtype expression during stages 8.2-12. Panels C and D shows the same embryo in an anterior (left), prosomal (middle) and opisthosomal (right) view. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-fz4-1* was first detected at stage 8.2 in a stripe domain (arrowhead) at the posterior of the O2 segment (arrow) and in a stripe (chevron) at the anterior portion of the SAZ (A). At stage 9.1, faint *Pt-fz4-1* expression can be detected in the mesoderm of the forming limbs and in circular domains in the precheliceral lobes (arrows) (B). *Pt-fz4-1* is strongly expressed in the limb mesoderm, the ventral neuroectoderm and the head lobes (arrows) at stage 9.2 (C). *Pt-fz4-1* expression continues in the limbs and the opisthosomal mesoderm (D). The expression in the head lobe is restricted to the anterior border and the labrum area (arrow, D).
6.1.4  *Pt-fz4-2* expression in *Parasteatoda*

*Pt-fz4-2* expression arises as an anterior stripe at stage 6 in a similar domain and at a similar time point to *Pt-fz4-1* (see fig. 45 A, 45 A). However, compared to *Pt-fz4-1* (see fig. 45 A), the expression domain is initially narrower and does not become as broad at stage 8.1 (see fig. 46 B). Later, at stage 8.2, *Pt-fz4-2* is strongly expressed in the segmental grooves in the pro- and opisthosomal segments and in a ring around the future labrum (see fig. 46 C). The expression of *Pt-fz4-2* retracts to the dorsal periphery of each segment at stage 9.2 and the domain at the labrum becomes more defined (see fig. 46 C). *Pt-fz4-2* is strongly expressed in the limb and opisthosomal mesoderm at stage 12 (see fig. 46 D), and at this stage is still expressed in the labrum and also becomes apparent in a specific area anterior to this structure (see fig. 46 D). However, *Pt-fz4-2* expression was not detected in the SAZ at any of the observed stages (see fig. 46 C-E).
Figure 46 | *Pt-fz4-2* wildtype expression during stages 6-12. Panels C,D,E show the same embryo respectively in an anterior (left), a prosomal (middle) and an opisthosomal (right) view. In all panels anterior is to the left and embryos are counterstained with DAPI. *Pt-fz4a* is first expressed in an anterior stripe domain at stage 6 (A). At stage 7 the expression domain becomes slightly broader (B). At stage 8.2, *Pt-fz4b* is expressed in pro- and opisthosomal segmental grooves and in a ring domain around the forming labrum (Lb, arrow) area (C). The expression of *Pt-fz4b* is retracted to the dorsal periphery of each segment at stage 9.2 and the domain at the labrum area (Lb, arrow) becomes more defined (C). Further, *Pt-fz4a* is strongly expressed in the limb and opisthosomal mesoderm at stage 13 (D). At the anterior, *Pt-fz4a* is continuously expressed in the labrum (Lb, arrow) and additionally appears in the stomodaeum (Sto, arrow)(D).
Summarising the results of the frizzled receptor expression analysis in *Parasteatoda*, while *Pt-fz2* and *Pt-fz4-2* are expressed in pattern possibly consistent with a role in segmentation, only *Pt-Fz1* out of the four frizzled genes was expressed in the SAZ during any of the stages analysed.

### 6.2 Secreted frizzled-related proteins in *Parasteatoda*

Secreted frizzled-related proteins (Sfrp) have been identified as Wnt signaling antagonists in vertebrates where they play a major role in embryonic development (Chapman et al., 2004; Esteve and Bovolenta, 2006; Leimeister et al., 1998; Rattner et al., 1997). Sfrps contain a frizzled-like CRD domain at their amino-terminal end, but lack the characteristic Frizzled trans-membrane domain, which suggests that they are secreted (Rattner et al., 1997).

In the *Parasteatoda* genome, a single secreted frizzled-related protein (*Pt-Sfrp*) was identified (Hilbrant and McGregor unpublished data) (see fig. 47).

<table>
<thead>
<tr>
<th>Pt-Sfrp</th>
<th>PSCVDIPENLTLCGIGTQMRPLHLDDHTMVEVSQQAGSWVPLNIECHPDTQLFLCSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm-Sfrp5</td>
<td>.T.....R..M....D....K........................................S.....F,.LK..S...........</td>
</tr>
<tr>
<td>Smim-Sfrp5</td>
<td>.T..M..............................</td>
</tr>
<tr>
<td>Pt-Sfrp</td>
<td>FSPVCLDRPIYPCRSLCDKVRAGCESRMQAYGFPPDMVKCDKFPVDNDMCISVQANANTE</td>
</tr>
<tr>
<td>Sm-Sfrp5</td>
<td>.........................................EA...QG...G...RV...Y....FLR...E...L.....TA.SGKS.A</td>
</tr>
<tr>
<td>Smim-Sfrp5</td>
<td>.........................................EA..QK...G...R............R.....I...........S...G</td>
</tr>
</tbody>
</table>

Figure 47 | Alignment of the Sfrp frizzled-like CRD domain. Identical aa are represented with dots and sequences are in order of similarity identified in protein BLAST. *Parasteatoda tepidariorum* (*Pt*), *Strigamia maritima* (*Sm*), *Stegodyphus mimosarum* (*Smim)*.

Previous characterisation of the structure and function of Sfrps was predominantly carried out in vertebrates, with representatives identified in a few
invertebrates and evidence for Sfrps missing in arthropods (Bovolenta et al., 2008).

However recently, five trans-membrane frizzled-receptors, as well as other frizzled-related genes, like a secreted Frizzled-related protein were also identified in the *Strigamia maritima* genome (Chipman et al., 2014).

### 6.2.1 Expression of *Pt-Sfrp* in *Parasteatoda*

*Pt-Sfrp* expression was first detected in a broad stripe at the anterior of the germ band at around stage 7 (see fig. 48 A), similarly to that described above for *Pt-fz2* and *Pt-fz4-2* (see fig. 44 A and 46 A). Later, *Pt-Srfp* is expressed in a broad stripe in the anterior SAZ, which resolves in ectodermal strips of the segmental grooves of prosomal and opisthosomal segments, but expression is absent from the midline (see fig. 48 B-D). The strong segmental expression continued until stage 10.2, the latest stage observed (see fig. 48 F-I).
Figure 48 | *Pt-Sfrp* wildtype expression at stage 8.2. Panels B-E and F-I show the same embryo respectively in an anterior (B), a prosomal (C,F), an opisthosomal (D,G,H) and a side view (E,I). In all panels anterior is to the left and embryos A-E are counterstained with DAPI. *Pt-Sfrp* expression commences in a broad anterior stripe (A). Later *Pt-Sfrp* expression is restricted to the lateral parts of the segmental groove (dashed lines in C) and in a broad band in anterior SAZ (dashed lines in D). At stage 10.2 *Pt-Sfrp* continues to be restricted to the lateral ectoderm of the segmental grooves (F-I).
6.3 Discussion

A previous study confirmed that there are four subfamilies of frizzled genes in metazoans (Schenkelaars et al., 2015). Four frizzled genes (Pt-fz1, Pt-fz2, Pt-fz4a and Pt-fz4b) were identified previously in Parasteatoda (Janssen et al., 2015) (see fig. 48), but it appears that fz3 has been lost in this spider and there has been a duplication of fz4 (Janssen et al., 2015).

The phylogenetic analysis including several panarthropod species (Tribolium castaneum (Tc), Zootermopsis nevadensis (Zn), Glomeris marginata (Gm), Strigamia maritima (Sm), Parasteatoda tepidariorum (Pt), Pholcus phalangoides (Pp), Stegodyphus mimosarum (Stm), Ixodes scapularis (Is), Mesobuthus martensii (Mm) and Euperipatoides kanangrensis (Ek)) confirmed four Frizzled receptor subfamilies, reported in metazoans previously (Janssen et al., 2015; Schenkelaars et al., 2015). Moreover it could be shown that Gm, Sm, Mm, Zn, Pp assemble in a Frizzled 3 cluster, whereas Fz3 appears lost in Pt, Is and Tc (Janssen et al., 2015). Interestingly, duplications of the Frizzled 4 subfamily could be found for two other spiders (Pt, Stm, ) and a scorpion (Mm), but not for the third spider (Pp) included in the analysis. Tree from (Janssen et al., 2015).
Figure 49 | Phylogenetic analysis of the frizzled receptors. Maximum likelihood tree of frizzled receptor amino acid sequences among metazoa. The bootstrap values from the maximum likelihood analysis are given at the nodes. Included species: *Tribolium castaneum* (Tc), *Zootermopsis nevadensis* (Zn), *Glomeris marginata* (Gm), *Strigamia maritima* (Sm), *Parasteatoda tepidariorum* (Pt), *Pholcus phalangoides* (Pp), *Stegodyphus mimosarum* (Stm), *Ixodes scapularis* (Is), *Mesobuthus martensii* (Mm) and *Euperipatoides kanangrensis* (Ek). Provided by Maarten Hilbrant for (Janssen et al., 2015).
6.3.1 Expression analysis of the *Parasteatoda* frizzled genes

Taken together, the analysis of the expression patterns of the Frizzled genes in *Parasteatoda* suggest they are involved in neuroectoderm development, segment border formation and maintenance and development of anterior structures (see figs. 43 - 46). Comparing these expression patterns among arthropods provides some useful insights into the roles of these genes and their evolution.

*Fz1* is expressed ubiquitously in embryos of *Parasteatoda*, the millipede *Glomeris*, and the onychophoran *Euperipatoides* as well as *Drosophila* and *Tribolium*. However, *fz1* expression can be observed in a segmental pattern in *Parasteatoda* and *Euperipatoides* at later stages. This suggests that *Fz1* could be involved in segmentation across panarthropods (Beermann et al., 2011; Janssen et al., 2015; Muller et al., 1999; Park et al., 1994a). Although this is a bit speculative when inferred from ubiquitous expression in the absence of functional data.

*Fz2* expression in *Parasteatoda* and *Glomeris* resembles expression in *Drosophila* and *Tribolium*, which starts out as a broad anterior domain and progresses into expression in segmental stripes (Beermann et al., 2011; Muller et al., 1999). Therefore, it has been suggested that *Fz2* might also be involved in segmentation in *Parasteatoda* and *Glomeris*, but not across panarthropods, since no segmental expression was detected in *Euperipatoides* (Janssen et al., 2015).

In the case of *Fz4*, a single copy was identified in both *Glomeris* and *Euperipatoides* (Janssen et al., 2015) (see fig. 45 & 46 ). Comparison of *Fz4* expression domains between these two species and the *Parasteatoda*
paralogs suggest that they perform various functions including nervous system development, segmentation and limb development (Janssen et al., 2015). The two fz4 paralogs in Parasteatoda show similar expression in the labrum and the walking legs (Janssen et al., 2015). While Parasteatoda fz4-1 is expressed early in the developing nervous system and the head lobes, Pt-fz4-2 exhibits specific segmental expression in prosomal and opisthosomal segments (Janssen et al., 2015). Generally, Pt-fz4-1 appears to be expressed more broadly, compared to the restricted expression of Pt-fz4-2, which might indicate subfunctionalization of those duplicated genes (Force et al., 1999; Lynch and Force, 2000).

6.3.2 Investigating Frizzled function in the spider

It has been hypothesized that frizzled receptors act redundantly or require combinatorial action. In Tribolium for example, only the combined knockdown of fz1 and fz2 causes germ band phenotypes, whereas fz2 RNAi does not have an effect and fz1 knockdown leads to limb malformations (Beermann et al., 2011). Individual pRNAi knockdown of the four Parasteatoda frizzled genes showed no detectable effect (data not shown). Hence, to obtain a better understanding of the function of frizzled receptors in Parasteatoda, double or even triple RNAi against different combinations of frizzled receptors should be undertaken.
Another interesting aspect of frizzled receptor function concerns which Wnt ligands bind to each of them. To date, there is no experimental evidence about which Wnt ligand binds to which Frizzled receptor in *Parasteatoda* and this would be particularly interesting to know with respect to segmentation. *Wnt5, Wnt7-1, Wnt8* and *Wnt11-2* are all expressed in the SAZ in *Parasteatoda* (Janssen et al., 2010), while *Pt-fz1* is expressed ubiquitously and *Pt-fz4a* and *Pt-fz4b* expression is only observed at the anterior border of the SAZ at stage 9 (see figs. 45 and 46 D). This suggests that *Pt-fz1* is the receptor used in the SAZ with perhaps *Pt-fz4-1* and *Pt-fz4-2* also acting during formation of some segments. To help understand these potential roles and interaction better, it would be useful to characterise in detail where each Wnt ligand protein is expressed perhaps by tagging them using CRISPR/Cas9.

### 6.3.3 Sfrp in *Parasteatoda*

In humans five Sfrps have been identified (SFRP 1-5), which are also present in all vertebrates (Bovolenta et al., 2008). Additionally, non-mammalian vertebrates like *Xenopus*, *zebrafish* and chicks exhibit another subgroup (Sizzled, Crescent, Tlc), which is similar in sequence to the human SFRP1/2/5 cluster (Bovolenta et al., 2008). In invertebrates, Sfrp homologs have been discovered in the purple sea urchin (Lapraz et al., 2006), the nematode *Caenorhabditis elegans* (Bovolenta et al., 2008), the sea squirt *Ciona intestinalis* (Hino et al., 2003) and in the sponge *Lubomirskia baicalensis* (Adell et al., 2007), which indicates the ancient origin of this signaling molecule family. Although, initially believed to be lost in arthropods, based on
the lack of Sfrps in the *Drosophila* genome, Sfrp homologs have also been discovered in the milipede *Strigamia maritima* (Chipman et al., 2014) and the spider *Parasteatoda* (M. Hilbrant and A. McGregor).

It was thought that SFRPs act as Wnt signaling antagonists in vertebrates but they have in fact been shown to play different roles in vertebrate development, where they activate as well as inhibit Wnt-signaling in different processes (Bovolenta et al., 2008; Esteve et al., 2011; Leyns et al., 1997; Wang et al., 1997). Furthermore it was shown that Sfrps interact with frizzled receptors (Bafico et al., 1999) and each other to inhibit function (Yoshino et al., 2001).

To obtain a better understanding of the function and mechanism of Sfrps in arthropods, Sfrps in other arthropod species need to be identified and functionally tested. In the case of the *Parasteatoda* homolog, a more detailed time series could be carried out and RNAi against the *Pt-Sfrp* could be undertaken. As Sfrps have been shown to interact with frizzled receptors (Bafico et al., 1999), *Pt-Sfrp* RNAi knockdown should also be carried out in *Parasteatoda* and the effect on embryogenesis and the expression of other Wnt signaling components assayed.
7. General discussion

7.1. Functional division of the SAZ and interaction between Delta-Notch and Wnt8 signaling pathways

This PhD provides further evidence that the *Parasteatoda* SAZ is subdivided into a posterior domain with high *Pt-Wnt8* expression, and an anterior *Pt-Wnt8* domain with relatively lower *Pt-Wnt8* expression both of which are regulated by Delta-Notch signaling (Schonauer et al., 2016) (see fig. 49). I propose, that *Pt-Dl* expression, cyclically progressing from the posterior to the anterior SAZ and on to the nascent segments, is primarily responsible for *Pt-Wnt8* repression in the anterior. These alternating states of *Pt-Dl* expression and consequently *Pt-Wnt8* repression in the anterior and vice versa, might enable the differentiation of cells and thus facilitate subsequent formation of segments from the SAZ at regular intervals. Whereas *Pt-N*, with its continuous expression in the SAZ, might be responsible for the maintenance of *Pt-Wnt8* expression in the posterior SAZ.

A similar functional compartmentalisation of the GZ, the SAZ equivalent in the cockroach, could be shown in *Periplaneta*: *Pa-Dl* expression oscillates through the GZ via activation by *Pa-Wnt1* in the posterior and repression by *Pa-cad* in a broad domain in the anterior part (Chesebro et al., 2013). Only when *Pa-Dl* expression exceeds a certain threshold, anterior of the *Pa-cad* domain, is segmentation gene expression activated, ensuring the sequential formation of segments in the cockroach (Chesebro et al., 2013).
Whilst no other comprehensive description of Delta-Notch and Wnt signaling interplay regulating sequential segment formation has been reported in other arthropods, expression and/or function of components of the Delta-Notch and Wnt signaling pathway suggest that they are likely to be crucial for short germ segmentation more widely.

In the centipede *Strigamia*, oscillating *Sm-Dl* expression has been observed throughout posterior development including the transition from double-segmental to single segmental expression during trunk segment formation (Chipman and Akam, 2008). In the cricket *Gryllus*, as well as in the milkweed bug *Oncopeltus* and the flour beetle *Tribolium*, functional analysis of components of the Wnt signaling pathway confirmed a role in posterior segment formation, however, no involvement of Delta-Notch signaling in segmentation has yet been found in those insects (Angelini and Kaufman, 2005; Aranda et al., 2008; Bolognesi et al., 2008; Kainz et al., 2011; Miyawaki et al., 2004).

However, it is still unclear how the dynamic *Pt-Dl* and *Pt-N* expression is generated and how *Pt-Dl* activates *Pt-Wnt8* in the posterior and represses in the anterior SAZ. Indeed, the loss of *Pt-N* expression in *Pt-Dl* RNAi embryos potentially suggests auto-inhibitory mechanism of this signaling pathway. Investigating the regulatory interactions between *Pt-Dl* and *Pt-N* further, could also give insight into the dynamics of their expression (see Discussion in Chapter 3 for further detail).

Furthermore, the effect of *Pt-Dl* and *Pt-N* on downstream factors should be studied in more detail: whilst the effect of *Pt-Dl* and *Pt-N* on *Pt-Wnt8* appears
similar, differences in their wild-type expression patterns suggest that they might be responsible for different aspects of gene expression of the SAZ. To address this question, a more detailed time series of Pt-DI and Pt-N double in situ analysis is needed, to get more information about their relative expression patterns at different stages of posterior development and in different compartments of the germ band. In addition, the generation of Pt-DI RNAi clones and subsequent in situ hybridisation to assay Pt-N expression at different developmental stages would also be insightful. Hereby, the effect on Pt-N with the Pt-DI clone can be compared to interactions with the surrounding wild-type tissue. These observations might elucidate the regulation between Pt-DI and Pt-N in different compartments of the Parasteatoda SAZ and thereby explain the differential effect on Pt-Wnt8 expression in the posterior and anterior SAZ.

7.2. The regulation of pair-rule gene orthologues

I also demonstrated that Delta-Notch and Wnt signaling together with caudal are required for pair-rule gene expression in the spider. In addition, I also showed that Pt-cad is downstream of Pt-DI. However, Pt-cad does not appear to be sufficient for Pt-eve activation. I could also show that Pt-eve does not activate Pt-cad, which suggests that Pt-eve acts downstream of Pt-cad (see fig. 50). These findings confirm that the regulation and expression of the pair-rule genes investigated, is not achieved by a pair-rule gene circuit, exactly as described in Tribolium (Choe et al., 2006), but appear to be regulated by Delta-Notch/Wnt/Cad in parallel.
Whilst it is challenging to infer the molecular composition and structure of segmentation in the common ancestor of arthropods from studying individual components of a presumably complex GRN, a common principle can be identified in several arthropod representatives: observations in *Parasteatoda*, together with evidence from *Periplaneta, Tribolium* and *Gryllus*, allow the conclusion, that *even-skipped* regulation by *caudal*, directed by upstream signaling pathways is ancestral to all arthropods (Chesebro et al., 2013; El-Sherif et al., 2014; Pueyo et al., 2008; Schonauer et al., 2016; Shinmyo et al., 2005) (and see Discussion in Chapter 5).

Furthermore, expression of pair-rule genes in *Strigamia, Cupiennius* and *Parasteatoda* suggest that segments were added one by one ancestrally and the double segmental pattern, observed in *Drosophila* and during the addition of many of the trunk segments in *Strigamia* possibly represents convergent evolution in geophilomorph centipedes and insects (Brena and Akam, 2013; Chipman and Akam, 2008; Chipman et al., 2004; Choe et al., 2006; Damen, 2004; Damen, 2007; Damen et al., 2000; Davis et al., 2001; Frasch and Levine, 1987; Green and Akam, 2013; Janssen et al., 2011; Leite and McGregor, 2016; Patel et al., 1994; Sarrazin et al., 2012; Schonauer et al., 2016; Schoppmeier and Damen, 2005a).

The expression profile of *Pt-eve* and *Pt-run-1* exhibits an early expression onset and a single segmental pattern in the SAZ and forming segments. Also, both genes are regulated by *Pt-cad* amongst other factors. Taken together, these findings suggest that both genes act on the same hierarchical level and function as primary pair-rule genes in *Parasteatoda*. I would be interested to investigate the regulation of the other pair-rule genes. For example, I suggest
analysing \textit{Pt-odd-1} or \textit{Pt-slp} in \textit{Pt-eve} and \textit{Pt-run} eRNAi embryos to determine if expression is lost in the clone area.

In case of a negative result showing no change to \textit{Pt-odd-1} and \textit{Pt-slp} expression, one might have to consider knocking down \textit{Pt-eve} and \textit{Pt-run-1} at the same time, as one of the primary pair rule genes might be sufficient for \textit{Pt-odd-1} and \textit{Pt-slp} expression.
Figure 50 | Summary of the GRN of posterior development in *Parasteatoda*. In the posterior of the SAZ (hatched area), DI-N (orange) activates Wnt8 (green) expression to maintain cells in an undifferentiated state. Wnt8 is then required for dynamic expression of DI, which results in the formation of a stripe of DI expression in anterior SAZ cells (white background). Wnt8 and DI-N are also required to activate caudal (blue) expression. These factors activate eve and run-1 (both violet) expression. In anterior SAZ cells, DI then subsequently suppresses Wnt8 expression and in combination with caudal, eve and runt expression leads to segment formation. Arrowheads and flat arrows indicate activation and repression, respectively, although it is not known if these interactions are direct or whether additional factors are required. Also for simplicity, the regulation of Wnt8 by DI and N is depicted, rather than the regulation of DI and N expression on each other. This schematic representation of the SAZ of *Parasteatoda* does not depict a particular stage of development, but aims to highlight the differences in regulation between the anterior and posterior SAZ.
7.3. The Evolution of Segmentation

Our knowledge about arthropod segmentation to this date allows two different scenarios explaining the evolution of segmentation: namely that the common bilaterian ancestor was segmented and utilized a Delta-Notch/Wnt signaling based mechanism to generate segments sequentially. In this case, it could be argued that the lack of involvement of Delta-Notch signaling in segmentation in insects like Tribolium and Drosophila is a derived state of segmentation (Aranda et al., 2008).

Alternatively, Delta-Notch/Wnt-based segmentation as observed in vertebrates and arthropods like Parasteatoda and Periplaneta (and potentially other arthropods) could have evolved independently by co-option of signaling pathways or other factors. Evidence for such an evolutionary history might be that some factors are ‘plugged-in’ to the network differently: caudal, for example represses Delta in the GZ of the cockroach, whereas it has no effect on Pt-Dl expression in the spider (Chesebro et al., 2013; Schonauer et al., 2016).

Nevertheless, we have to bear in mind that we may so far have only examined in detail a small part of a presumably complex GRN consisting of numerous factors, intertwined by regulatory mechanisms, which ensure the correct expression at the right time, in the correct place. And whilst expression patterns give us a good indication about a potential role, only the functional analysis of the GRN components in other arthropods, and outgroups like Onycophorans (Janssen and Budd, 2013; Strausfeld et al., 2006) and Priapulids (Webster et al., 2006) as well as annelids is likely to provide...
sufficient information to address the question of evolution of segmentation in bilateria further.

7.4. Future directions to understand segment addition in *Parasteatoda*

*Parasteatoda* has proven to be an excellent model organism for studying arthropod segmentation, due to the easy access to embryos, their well described embryonic development, the gene knockdown techniques including the generation of somatic clones and the reliable mRNA staining protocol (Hilbrant et al., 2012). However, during the course of my work some additional questions arose that could be addressed in future experiments to provide a better understanding of the mechanisms of sequential segment addition in the spider.

The results showing that *Pt-cad* is not sufficient to activate *Pt-eve* in the posterior and most likely requires other unknown factors. This highlights the fact that there are certainly more components involved in regulating the formation of the SAZ, its maintenance and the subsequent formation of segments from this tissue. Therefore, an unbiased, non-candidate gene approach towards identifying other parts of the GRN of posterior segmentation is required. This could be carried out by preparing RNA-seq libraries of SAZ tissue from wild-type embryos and for example *Pt-Wnt8* RNAi knockdown embryos at different stages (before SAZ formation / when the SAZ has formed / after formation of the first segment). This would generate the expression profile of all the genes expressed at different stages of posterior development and those that are regulated by Wnt8 signaling; thus providing
new candidates for further expression and functional studies and broadening our understating of the GRN for posterior segment addition in *Parasteatoda*.

Another aspect of segmentation opened up during my PhD work, concerns the mesoderm. Previous work in the spider showed that the knockdown of *Pt-Dl* disrupts the equal formation of caudal meso- and ectoderm, through overexpression of the mesodermal determination gene *twist* (*Pt-twist*) and the lack of *Pt-cad* in the posterior (Oda et al., 2007). Note that previous analysis also showed that *Pt-twi* is involved in mesoderm development in the spider (Yamazaki et al., 2005). Intriguingly, I observed *Pt-twi* expression in the prosoma which suggests that cells delineate from one stripe domain and migrate to an anterior stripe (Schoenauer, unpublished). To better understand the development and segmentation of the mesoderm, I would attempt to fluorescently label and observe *Pt-twi* using CRISPR/Cas9 and Pt-Twi protein expression over the course of posterior development. In parallel, I would functionally analyse this gene and further using embryonic RNAi to investigate the regulatory interactions with the already known factors such as *Pt-Dl*.

As outlined earlier, the labelling of components of the SAZ could not only provide information on the gene expression dynamics, but would also allow the tracking of cell movements over the course of posterior development. It could be shown previously that there is not a significant rate of cell proliferation happening in the SAZ during elongation of the germ band (McGregor et al., 2008b). In *Tribolium*, fluorescently labelled clones of cells revealed differences in cell behaviour dependent on their location and
differences in the segment addition rate over the course of posterior development (Nakamoto et al., 2015). By labelling components of the GRN of segmentation, the protein localization in correlation with development time, the timing of segment addition and cell movements in the SAZ could be elucidated. This would provide a better understanding of cell behaviour underlying SAZ function to compliment our genetic insights.
8. References


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