

The Wnt and Delta-Notch signalling pathways interact to direct pair-rule gene expression via *caudal* during segment addition in the spider *Parasteatoda tepidariorum*

Anna Schönauer¹, Christian L. B. Paese¹, Maarten Hilbrant^{1,2}, Daniel J. Leite¹, Evelyn E. Schwager^{1,3}, Natália Martins Feitosa⁴, Cornelius Eibner⁵, Wim G. M. Damen⁵ and Alistair P. McGregor^{1*}

¹ Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, OX3 0BP, UK.

² Present address: Institute for Developmental Biology, University of Cologne, Zùlpicher Str. 47b, 50674 Cologne, Germany

³ Present address: Department of Biological Sciences, University of Massachusetts Lowell, 198 Riverside St., Lowell, MA 01854, USA

⁴ Laboratório Integrado de Ciências Morfofuncionais, Universidade Federal do Rio de Janeiro- UFRJ/NUPEM-Campus Macaé

⁵ Department of Genetics, Friedrich-Schiller-University Jena, Philosophenweg 12, 07743 Jena, Germany

*Corresponding author: amcgregor@brookes.ac.uk

Keywords: segmentation, spiders, Wnt signalling, Delta-Notch signalling, *caudal*, *even-skipped*.

Summary

In short germ arthropods, posterior segments are added sequentially from a growth zone or segment addition zone (SAZ) during embryogenesis. Studies in spiders such as the common house spider, *Parasteatoda tepidariorum*, have provided insights into the gene regulatory network (GRN) that underlies the development of the SAZ, and revealed the involvement of two important signalling pathways. It was shown that *Wnt8* maintains a pool of undifferentiated cells in the SAZ, but this ligand is also required for dynamic *Delta* (*DI*) expression associated with the formation of new segments. However, it remains unclear how these pathways interact during SAZ formation and subsequently regulate segment addition. Here we show that Delta-Notch signalling is required for *Wnt8* expression in posterior SAZ cells, but represses the expression of this Wnt gene in anterior SAZ cells. We also found that these two signalling pathways are required for the expression of the spider orthologues of the segmentation genes *even-skipped* (*eve*) and *run-1* (*run-1*), at least in part via the transcription factor encoded by *caudal* (*cad*). Moreover, it appears that dynamic expression of *eve* in this spider does not require a feedback loop with *run-1*, as is found in the pair-rule circuit of the beetle *Tribolium*. Taken together, our results suggest that the development of posterior segments in *Parasteatoda* is directed by dynamic interactions between *Wnt8* and Delta-Notch signalling that are read out by *cad*, which is necessary but not sufficient to regulate the expression of the pair-rule genes *eve* and *run-1*. Our study therefore provides new insights towards better understanding the evolution and developmental regulation of segmentation in other arthropods including insects.

Introduction

The regulation of arthropod segmentation is best understood in *Drosophila melanogaster*, which employs a well-characterised cascade of transcription factors to generate its segments almost simultaneously along the antero-posterior axis (reviewed in Pankratz and Jäckle, 1993; Peel et al., 2005). In contrast to this long germ mode of segmentation, most insects and other arthropods exhibit a short germ mode of segmentation, during which only a few anterior segments are generated simultaneously and subsequently a variable number of posterior segments are added sequentially from a posterior growth zone or SAZ (Davis and Patel, 2002; Tautz, 2004; Peel et al., 2005; McGregor et al., 2009). Note, however, that these modes of segmentation may not always be discrete since a mixed mode of segmentation has recently been proposed for *Nasonia vitripennis* (Rosenberg et al., 2014).

Comparative studies have shown that aspects of the *Drosophila* segmentation cascade are also found in short germ arthropods, indicating that these were probably features of the regulation of segmentation in the arthropod common ancestor (Peel et al., 2005). Firstly, there is evidence that *hunchback* and *Distal-less* perform gap gene like functions during formation of the prosomal segments of spiders (Schwager et al., 2009; Pechmann et al., 2011). Secondly, the orthologues of *Drosophila* pair-rule genes are also expressed in the SAZ and segments of short germ arthropod embryos, which is consistent with roles in segmentation in these animals, although it is likely that these genes were expressed in single rather than double segmental periodicity ancestrally in arthropods (Frasch et al., 1987; Sommer and Tautz, 1993; Patel et al., 1994; Damen et al., 2000; Davis et al., 2001; Dearden et al., 2002; Chipman et al., 2004b; Damen et al., 2005; Schoppmeier and Damen, 2005b; Choe et al., 2006; Damen, 2007; Mito et al., 2007; Chipman and Akam, 2008; Janssen et al., 2011; Sarrazin et al., 2012; Brena and Akam, 2013; Green and Akam, 2013). Finally, the expression

and function of segment polarity genes are highly similar across arthropods (Damen, 2002; Hughes and Kaufman, 2002).

In contrast to the regulation of segment formation in *Drosophila* and possibly other holometabolous insects, the formation of the SAZ and generation of posterior segments in many short germ arthropods is regulated by a probably ancestral GRN that includes the Delta-Notch signalling pathway (Stollewerk et al., 2003; Schoppmeier and Damen, 2005a; Oda et al., 2007; Chipman and Akam, 2008; Pueyo et al., 2008), together with Wnt signalling (Bolognesi et al., 2008; McGregor et al., 2008b) and *caudal* (*cad*) (McGregor et al., 2009; Chesebro et al., 2013). Further understanding the underlying interactions in this GRN and how it directs segmentation in short germ arthropods can provide much needed new insights into the evolution of these processes.

The spider *Parasteatoda tepidariorum* (formerly *Achaearanea tepidariorum*) has proven to be an excellent chelicerate model for studying segmentation in short germ arthropods (Schwager et al., 2015). This success has been facilitated by detailed descriptions of its early embryogenesis (Akiyama-Oda and Oda, 2003; Mittmann and Wolff, 2012), the establishment of tools to study gene expression and gene function (Akiyama-Oda and Oda, 2006; McGregor et al., 2008a; Kanayama et al., 2010; Kanayama et al., 2011; Hilbrant et al., 2012), as well as the availability of embryonic transcriptomic resources (Posnien et al., 2014).

In *Parasteatoda* prosomal and opisthosomal segmentation appear to be regulated by different mechanisms. The formation of prosomal segments requires traveling waves of *hh* and *otd* expression and more posteriorly *hb* and *Dll* (Pechmann et al., 2009; Schwager et al., 2009; Kanayama et al., 2011; Pechmann et al., 2011); whereas the Wnt8 (*Pt-Wnt8*) and Delta-Notch signalling pathways are required for formation of the SAZ and development of opisthosomal segments (Oda et al., 2007; McGregor et al., 2008b). It was shown previously that knockdown of *Pt-Wnt8* or *Pt-Delta* (*Pt-Dl*) using parental RNAi results in strongly

reduced expression of *Pt-cad* expression and gives rise to truncated embryos with malformed or even an absence of posterior segments (Oda et al., 2007; McGregor et al., 2008b). This suggested that these signalling pathways might act via *Pt-cad* during segment addition (Oda et al., 2007; McGregor et al., 2008b). These effects may also be explained, at least in part, by the fact that *Pt-Wnt8* is required for the dynamic expression of *Pt-Dl* that is associated with the formation of new posterior segments (McGregor et al., 2008b).

However, in *Parasteatoda* and other short germ arthropods that have been shown to employ Wnt/Delta-Notch/Cad, it is not understood how these key factors interact with each other. Furthermore, it is not known how the expression of putatively downstream segmentation genes like, for example *even-skipped*, is regulated compared to other arthropods. In *Drosophila*, *eve* is regulated by a combination of maternal and gap factors, while in *Tribolium*, *eve* expression is regulated by *caudal* and other pair-rule genes that likely operate in a circuit (Small et al., 1991; Small et al., 1992; Fujioka et al., 1996; Choe et al., 2006; El-Sherif et al., 2014), most likely downstream of Wnt signalling (Oberhofer et al., 2014). Therefore, to better understand the regulation of segment formation in short germ arthropods, we further investigated the regulatory interactions between *Pt-Dl*, *Pt-Notch* (*Pt-N*), *Pt-Wnt8* and *Pt-cad* and studied the expression and regulation of the *Parasteatoda* orthologues of the *Drosophila* pair-rule genes *eve* (*Pt-eve*) and *runt* (*Pt-run-1*) during early embryogenesis in this spider.

We found that Delta-Notch signalling is required for the activation of *Pt-Wnt8* expression in posterior SAZ cells. However, knockdown of *Pt-Dl* or *Pt-N* results in increased expression of *Pt-Wnt8* in anterior SAZ cells. Therefore, Delta-Notch signalling is required to suppress expression of *Pt-Wnt8* in anterior SAZ cells presumably to facilitate segment formation from the undifferentiated pool of cells probably maintained by this Wnt gene. We also found that knockdown of *Pt-Wnt8* or *Pt-Dl* results in the loss of *Pt-eve* and *Pt-run-1*

expression. This can be explained by the loss of *Pt-cad* expression when these pathways are perturbed because we show that knockdown of *Pt-cad* expression alone in the SAZ inhibits *Pt-eve* and *Pt-run-1* expression, although *Pt-cad* does not appear to be sufficient to activate these genes. In addition, we observed that *Pt-eve* does not appear to regulate *Pt-runt-1* (*Pt-run-1*) expression or vice versa. This finding suggests that while the pair rule gene orthologs in *Parasteatoda* may still form a regulatory circuit, it cannot be based on exactly the same regulatory interactions that have been identified in *Tribolium* (Choe et al., 2006). Therefore, segment addition in *Parasteatoda* appears to be directed by dynamic interactions between Wnt8 and Delta-Notch signalling in the SAZ that results in the activation of *Pt-cad*, which is necessary for the regulation of the expression of the pair-rule gene orthologues *Pt-eve* and *Pt-run-1*.

Results

Posterior development in Parasteatoda is regulated by dynamic interactions between the Delta-Notch and Wnt8 signalling pathways

In *Parasteatoda*, formation of the SAZ and production of segments from this tissue require both Wnt8 and Delta-Notch signalling (Oda et al., 2007; McGregor et al., 2008b). We previously showed that *Pt-Dl* expression is established normally in *Pt-Wnt8* knockdown embryos, but subsequently fails to successively clear from the posterior (McGregor et al., 2008b). This suggests that *Pt-Wnt8* is necessary for dynamic *Pt-Dl* expression during posterior development in *Parasteatoda*.

During stage 6, after *Pt-Dl* expression has cleared from posterior SAZ cells, this gene is expressed in a salt and pepper pattern juxtaposed to a more diffuse domain in anterior SAZ cells (Figs 1A and S1A). We noticed that *Pt-Wnt8* expression is weaker in anterior SAZ cells, where it overlaps with the diffuse *Pt-Dl* expression domain, compared to the stronger expression of *Pt-Wnt8* detected in posterior SAZ cells (Fig. 1B) (McGregor et al., 2008b). We therefore investigated whether *Pt-Dl* is involved in the regulation of *Pt-Wnt8*. We found that knockdown of *Pt-Dl* using parental RNAi (pRNAi) results in the loss of *Pt-Wnt8* expression in the posterior of the SAZ, but conversely gives rise to stronger *Pt-Wnt8* expression in the anterior SAZ cells (Fig. 1C).

Pt-N is expressed in similar pattern to *Pt-Dl* in the SAZ at stage 6, but *Pt-N* expression is then maintained in a more diffuse pattern during stage 7 with slightly stronger expression observed in the newly forming segment (Fig. S2) Note that *Pt-Dl* and *Pt-N* may initially be expressed in different cells in the SAZ since knockdown of one leads to more diffuse expression of the other at stage 6, suggesting they may inhibit each others expression at this stage (Fig. S2; Oda et al., 2007).

We then tested whether *Pt-Wnt8* expression also requires *Pt-N* we found that knockdown of *Pt-N* using parental RNAi has a very similar effect to knockdown of *Pt-Dl* on the expression of *Pt-Wnt8* (Fig. S3). This suggests that Delta-Notch signalling is required 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. It is also possible that *Pt-N*, but not *Pt-Dl* helps to maintain *Pt-Wnt8* expression in the SAZ, because the expression of *Pt-N* persists in the SAZ while *Pt-Dl* expression is cyclical (Fig. S2).

Pt-cad is not required for dynamic *Pt-Dl* expression in the SAZ

It was previously shown that *Pt-Dl* and *Pt-Wnt8* are required for the establishment of *Pt-cad* expression in the SAZ (Oda et al., 2007; McGregor et al., 2008b). Like *Pt-Dl*, *Pt-cad* also exhibits dynamic expression in the SAZ, forming stripes of expression in each nascent segment (Fig. S4) (Oda et al., 2007). This expression of *Pt-cad* is slightly out of phase with *Pt-Dl* expression, but the expression of these two genes overlaps in some cells (Fig. S1A).

We then asked if *Pt-cad* regulates *Pt-Dl* in the SAZ. Since *Pt-cad* pRNAi has no discernable effect on morphology or gene expression despite repeated attempts (data not shown), we instead performed embryonic RNAi (eRNAi), where dsRNA is injected into single blastomeres at the 8-16 cell stage, leading to RNAi effects in clones of the injected blastomere (Kanayama et al., 2010; Kanayama et al., 2011). We found that eRNAi against *Pt-cad* results in strongly reduced levels of *Pt-cad* transcripts in eRNAi clones of SAZ cells compared with *Pt-cad* expression in SAZ cells neighbouring the clone (n = 5) (Fig. S5A). However, the level of *Pt-Dl* expression was unaffected in *Pt-cad* knockdown clones in the SAZ that overlapped with cells that express both *Pt-Dl* and *Pt-cad* in wild-type embryos (n =

14) (Fig. S1B). This suggests that *Pt-cad* is not involved in the regulation of *Pt-Dl* during posterior development in *Parasteatoda*.

Pt-eve is expressed in the SAZ and opisthosomal segments

To better understand segment addition in *Parasteatoda*, we next characterised the expression of *Pt-eve* during early embryogenesis. We observed that *Pt-eve* is initially expressed in a small oval domain of approximately 20 cells in the SAZ at stage 6 (Fig. S6A). This expression domain then increases in size (Fig. 2A), but concomitantly the centre clears to form a transient ring of expression (Fig. 2B). This ring of *Pt-eve* expression is broken by the apparent loss of transcripts in the most posterior cells (Fig. 2C), giving rise to a stripe of expression, approximately 3-5 cells wide, in the nascent O1 segment during stage 7 (Fig. 2D). At this stage, expression of *Pt-eve* is again observed in a circular domain in the most posterior cells of the SAZ (Fig. 2D), which again clears centrally (Fig. 2E) to form a second stripe in the presumptive O2 segment. At the same time, the older stripe of *Pt-eve* expression in O1 begins to narrow and expression decreases (Fig. 2F).

Subsequently *Pt-eve* expression undergoes similar dynamic cycles of strong expression in the posterior SAZ cells followed by the clearance of expression from this region and expression in the forming segments in anterior SAZ cells. As *Pt-eve* stripes form in nascent segments, the expression in the older, more anterior, segments fades. For example, during formation of O3 (Fig. 2G), *Pt-eve* expression is observed in O2 and O3 and the SAZ but is no longer detected in O1. De novo *Pt-eve* expression is seen in the developing central nervous system in older anterior segments (Fig. 2H). In summary, the expression of *Pt-eve* is consistent with the involvement of this gene in regulating formation of all segments posterior to and including O1, as well as differentiation of the nervous system.

Expression of Pt-eve in the SAZ is dependent on Wnt8 and Delta-Notch signalling

Since Wnt8 and Delta-Notch signalling are required for the formation of the SAZ and the generation of posterior segments, we tested if these pathways are required for *Pt-eve* expression. Compared to wild-type embryos (Fig. 3A), we did not detect *Pt-eve* expression in *Pt-Dl* pRNAi embryos (Fig. 3B). Similarly we found that *Pt-eve* expression was strongly reduced in *Pt-Wnt8* pRNAi embryos (Fig. 3C). Therefore both *Pt-Wnt8* and *Pt-Dl* are required for *Pt-eve* expression in the SAZ. However, since knockdown of *Pt-Dl* and *Pt-Wnt8* also results in the loss of *Pt-cad* expression (Oda et al., 2007; McGregor et al., 2008b), the effect of knocking down these pathways on *Pt-eve* expression might be an indirect effect caused by loss of *Pt-cad* expression.

Pt-cad is necessary but not sufficient for Pt-eve expression

To investigate if *Pt-cad* could regulate *Pt-eve* expression and/or vice versa, we first carried out double *in situ* hybridisations to compare the expression of these two genes relative to each other during posterior development (Figs 4A, A', C, C' and S6). *Pt-cad* and *Pt-eve* expression are initially detected at early stage 6 when cells appear to first express *Pt-cad* alone and then express both genes in a small oval shaped domain (Figs 3A, 4A and S6A, A'). Subsequently, *Pt-eve* and *Pt-cad* expression expand into an overlapping circular domain in the SAZ, but *Pt-cad* expression appears to persist in the more posterior cells from which expression of *Pt-eve* has cleared (Fig. 4A, A'). These two genes continue to be expressed in a similar fashion during the subsequent addition of segments. For example at stage 7, both *Pt-eve* and *Pt-cad* are expressed in overlapping stripes in the nascent O1 segment: *Pt-eve* is expressed exclusively in the anterior-most row of cells whereas *Pt-cad* is also expressed solely in approximately two rows of the most posterior cells (Fig. 4C). At this stage a new domain of

overlapping expression of *Pt-eve* and *Pt-cad* can also be observed in posterior SAZ cells (Fig. 4C).

The relative expression patterns of *Pt-cad* and *Pt-eve* suggest that there might be a regulatory interaction between these two genes during posterior development. We therefore performed eRNAi and generated twelve independent *Pt-cad* eRNAi clones in cells that overlapped with the normal expression domains of *Pt-eve* in stage 6 and 7 embryos. In all embryos, in which the *Pt-cad* eRNAi clone overlapped with the circular *Pt-eve* expression domain at stage 6, we observed that *Pt-eve* expression was completely lost or very strongly reduced (Fig. 4B). Later at stage 7, when *Pt-eve* expression is observed in posterior SAZ cells and in more anterior cells in a stripe corresponding to the nascent O1 segment, expression of *Pt-eve* was reduced in both expression domains that overlapped with *Pt-cad* eRNAi clones (Fig. 4D). These results suggest that *Pt-cad* is probably required for the activation and maintenance of *Pt-eve* expression during posterior development in *Parasteatoda*, however it is not clear whether this regulation is direct or indirect.

We then tested if *Pt-cad* expression is sufficient to activate *Pt-eve* expression by injecting capped *Pt-cad-eGFP* mRNA into blastomeres at the 16 cell stage and allowing them to develop until stage 5 (i.e. before *Pt-cad* and *Pt-eve* are normally expressed). We were able to detect clones of cells with nuclear GFP expression (Fig. S7), demonstrating that *Pt-cad* was expressed and able to localise to the nuclei ($n = 5$). However, we did not observe expression of *Pt-eve* in any of these cells indicating, that while *Pt-cad* expression is required for *Pt-eve* expression, it is not sufficient in these conditions (Fig. S7). Indeed, since some of these cells near the pole of the germ at this stage are likely to express *Wnt8* and *Dl*, this implies that an additional factor or factors are required to activate *Pt-eve* (Fig. S7).

Next we tested whether *Pt-eve* regulates *Pt-cad*. As is the case for *Pt-cad*, pRNAi against *Pt-eve* had no discernable effects on morphology or gene expression suggesting that

this approach does not work for this gene (data not shown). However, we were able to knockdown *Pt-eve* expression in clones of cells that overlapped with the normal expression of this gene using eRNAi (n = 4) (Fig. S5) (Kanayama et al., 2010). Using this approach we then assessed *Pt-cad* expression in *Pt-eve* eRNAi clones, and found that *Pt-cad* expression in both posterior SAZ cells and in the nascent O1 segment was unaffected in overlapping *Pt-eve* eRNAi cell clones (n = 16) (Fig. S5C, D), suggesting that *Pt-eve* does not regulate *Pt-cad*.

Pt-eve and *Pt-run-1* do not regulate each other

In the beetle *Tribolium*, pair-rule genes have been shown to function in a regulatory circuit (Choe et al., 2006). Central to this model is the production of dynamic stripes of *eve* expression that rely on a negative feedback loop whereby *eve* activates *runt* (*run*) which activates *odd-skipped*, which represses *eve* (Choe et al., 2006).

To investigate whether a similar circuit based on the regulation of *Pt-run-1* by *Pt-eve* could be involved in segment addition in *Parasteatoda*, we first assayed the expression of *Pt-run-1* compared to *Pt-eve*. Note that we identified one other *runt*-like gene in the transcriptome of *Parasteatoda*, but it is not expressed in a pattern consistent with roles in segmentation (data not shown).

We found that the expression of *Pt-run-1* commences during stage 6 (Fig. S8A), at approximately the same time that *Pt-eve* expression is first detected (Fig. 2A). Moreover, *Pt-run-1* and *Pt-eve* expression partially overlap in posterior and anterior SAZ cells at all stages assayed (Fig. S8). However, *Pt-eve* is expressed approximately three cell rows anterior to *Pt-run-1* in stripes in the anterior of the SAZ (Fig. S8G-I).

We then tested if *Pt-eve* is required for the expression of *Pt-run-1* using eRNAi. However, *Pt-run-1* expression appeared to be normal where it overlapped with *Pt-eve* RNAi cell clones in the SAZ compared to neighbouring cells (n = 12) (Fig. 5). This suggests that, in

contrast to the pair-rule circuit in *Tribolium*, in *Parasteatoda*, *Pt-eve* does not regulate *Pt-run-1* during segment addition. We also investigated if *Pt-run-1* is required for the activation of *Pt-eve*; however, knockdown of *Pt-run-1* using eRNAi had no effect on *Pt-eve* expression in the SAZ (n=10) (Fig. S9). Indeed, it appears that *Pt-run-1* is actually regulated in parallel to *Pt-eve* because RNAi knockdown of *Pt-Wnt8* and *Pt-Dl* by pRNAi and *Pt-cad* eRNAi all greatly reduced *Pt-run-1* expression (Fig. S10), which is similar to the effect on *Pt-eve* expression when these genes are knocked down (Figs 3 and 4).

Discussion

Wnt8 and Delta-Notch signalling direct Pt-eve and Pt-run-1 expression via Pt-cad

We have found that *Pt-eve* and *Pt-run-1* expression in the SAZ requires *Pt-cad* expression, which explains the loss of their expression in *Pt-Wnt8* or *Pt-Dl* knockdown embryos, because these signalling pathways are also required for *Pt-cad* expression (Oda et al., 2007; McGregor et al., 2008b). However, we also found that *Pt-cad* expression alone is probably not sufficient to activate *Pt-eve* expression. This supports the interpretation that *Pt-cad* expression is regulated by Wnt8 and Delta-Notch signalling and that these factors together activate the expression of *Pt-eve* and *Pt-run-1* (Fig. 6), although it remains possible that other currently unknown transcription factors that may or may not be regulated by Wnt8 and Delta-Notch signalling are also required.

Furthermore, since it does not appear that *Pt-eve* feeds back to regulate *Pt-cad* or that *Pt-Dl* expression requires *Pt-cad*, it is likely that the cyclical expression of segmentation genes, such as *Pt-eve* and *Pt-run-1*, and the production of segments from the spider SAZ, is driven by dynamic interplay between the Wnt8 and Delta-Notch signalling pathways (Fig. 6). This model is supported by our finding that Delta-Notch signalling is required to activate *Pt-Wnt8* in posterior SAZ cells and that *Pt-Wnt8* then facilitates dynamic expression of *Pt-Dl* in

the SAZ (Fig. 6). Our results suggest that Delta-Notch signalling then suppresses *Pt-Wnt8* expression in anterior SAZ cells possibly to allow the formation of segments from a pool of undifferentiated cells maintained by this Wnt ligand (McGregor et al., 2008b). However, it is still unclear how dynamic expression of *Pt-Dl* and *Pt-N* is generated and how Delta-Notch signalling activates *Pt-Wnt8* in posterior SAZ cells, but suppresses the expression of this Wnt ligand gene in anterior SAZ cells. Based on the effects of the reciprocal knockdown of *Pt-N* and *Pt-Dl* on the expression of the other in the *Parasteatoda* SAZ (this study and Oda et al., 2007), we hypothesise that this could involve auto inhibitory regulation in the Delta-Notch pathway (Kageyama et al., 2007) as well as other, still undiscovered, genes expressed in the SAZ of *Parasteatoda*.

There is functional evidence that similar genetic interactions to those that we have identified in *Parasteatoda* likely also regulate segmentation in other arthropods. In embryos of the cockroach *Periplaneta*, there is feedback between *Wnt1* and *Dl* in the SAZ, and *Wnt1* also activates *cad* to generate a signalling centre responsible for the generation of posterior segments (Chesebro et al., 2013). Unlike in *Parasteatoda*, however, *cad* represses *Dl* in the *Periplaneta* SAZ (Chesebro et al., 2013). Furthermore, it is likely that *Wnt1* is required for *cad* expression during posterior development in *Tribolium*, like it is in *Gryllus* (Shinmyo et al., 2005; McGregor, 2006; Oberhofer et al., 2014), and a recent study has shown that the graded expression of *Tc-cad* is required for the dynamic expression of *Tc-eve* (El-Sherif et al., 2014). This suggests that although there are differences in the regulation of segment addition among short germ arthropods, the regulation of *eve* by *cad*, probably directed by upstream signalling pathways, may have been used ancestrally in arthropods.

Evolution of the expression and interactions of pair-rule orthologues among arthropods

In *Parasteatoda*, *Pt-eve* and *Pt-run-1* are expressed in the SAZ and subsequently in stripes associated with the formation of all of the segments that are generated from this tissue i.e. O1 and all of the following posterior segments. This result is consistent with previous analysis of these and other pair-rule orthologues in the Central American wandering spider *Cupiennius salei* (Damen et al., 2000; Damen et al., 2005). Our data provide further evidence for differences in the regulation of prosomal and opisthosomal segments in spiders, whereby gap and pair-rule gene orthologues respectively direct the formation of segments in these tagmata (Damen et al., 2000; Damen et al., 2005; Pechmann et al., 2009; Schwager et al., 2009; Pechmann et al., 2011). Interestingly, this also indicates that the roles of *eve* and *run-1* in spiders is restricted to formation of more posterior segments than, for example, in the insects *D. melanogaster* and *Tribolium*, and the myriapods *Strigamia maritima* and *Glomeris marginata*, in which *eve* is expressed in a segmental pattern in four segments more anterior to O1/T2 (Frasch et al., 1987; Brown et al., 1997; Janssen et al., 2011; Brena and Akam, 2013). Moreover, while there is a hierarchy of primary and secondary pair-rule gene orthologues in arthropods (Damen, 2007), our study further exemplifies that the register of the expression of these genes has diverged among these animals: *eve* and *run* expression overlap in forming segments in *Glomeris* like in *Parasteatoda*, but they are out of phase in *Strigamia* and *Drosophila* (Green and Akam, 2013).

The requirement of *eve* for *run* expression in *Tribolium* is a key regulatory step in the pair-rule circuit that underlies segmentation in this beetle (Choe et al., 2006). However, in *Parasteatoda*, we did not find a requirement of *Pt-eve* for the expression of *Pt-run-1* (or vice versa), which suggests that if this spider, like *Tribolium*, also employs a pair-rule circuit, it is composed of different genetic interactions. It also remains possible that a pair-rule circuit may not be a feature of spider segmentation, possibly because these arthropods employ Delta-Notch in combination with Wnt signalling instead. Thus a pair-rule circuit might be a derived

feature of segmentation in arthropods that no longer depends on Wnt with Delta-Notch signalling. Indeed, there is no evidence that Delta-Notch signalling regulates segmentation in *Tribolium* although some genes including *hairy* are expressed in pattern consistent with a role in segmentation (Tautz, 2004; Aranda et al., 2008).

Segmentation in the common ancestor of arthropods?

Although it is difficult to infer the role of genes and pathways and architecture of gene regulatory networks that may have existed in a common ancestor from the expression and function of a few genes and pathways in divergent extant organisms, an understanding is emerging of how segment addition may have been regulated in the arthropod common ancestor. Studies of segmentation in a range of arthropods including spiders, the myriapod *Strigamia* and *Periplaneta* suggest that a Wnt/Delta-Notch/Cad based system acting upstream or possible in parallel to pair-rule gene orthologues was probably the ancestral mechanism in arthropods (Chipman et al., 2004a; Chipman and Akam, 2008; McGregor et al., 2008b; Pueyo et al., 2008; McGregor et al., 2009; Brena and Akam, 2013; Chesebro et al., 2013). Furthermore, the work on spiders and *Strigamia* indicates that in this ancestor, segments were probably added with single segment periodicity (e.g. this work; Brena et al., 2013).

Conclusions

We have found that, during segment addition in *Parasteatoda*, the dynamic expression of *Pt-eve* and *Pt-run-1* requires *Pt-cad*, whose expression is a read-out of a dynamic interplay between the Wnt and Delta-Notch signalling pathways. This provides new insights into the transition between the formation of the SAZ and the production of segments from this tissue in the spider, and the evolution of this key developmental process among arthropods. Future work in this spider will allow us to determine if *Pt-eve* and *Pt-run-1* are direct targets of *Pt-*

cad, precisely how Delta-Notch and Wnt signalling interact at the cellular and molecular level, and investigate the possible involvement of other genes during segment addition.

Materials and Methods

Embryo collection, fixation and staging

Embryos were collected from adult female *Parasteatoda tepidariorum* from our laboratory culture in Oxford that was founded with spiders from Göttingen (Germany). The spider culture was kept at 25°C and embryos of stages 5 to 9 fixed as described in Akiyama-Oda and Oda (2003). Embryos were staged according to Mittmann and Wolff (2012). Note that these stages were chosen for this study because the SAZ develops from the caudal lobe, which is formed during stages 5 and 6. The first segment (O1) is added from the SAZ during stage 7, and subsequent segments are added from stage 8 onwards.

In situ hybridisation

Total RNA was extracted from a mixture of embryonic stages 5 to 9 using the RNeasy Lipid Tissue Mini Kit (Qiagen). cDNA was synthesised from total RNA with the QuantiTect Reverse Transcription Kit (Qiagen). *Pt-cad* (AB096075), *Pt-eve* (Locus_7056), *Pt-Dl* (AB287420), *Pt-N* (AB287421), *Pt-Wnt8* (FJ013049), *Pt-run-1* (Locus_15496) and *Pt-run-2* (Locus_12769) sequences were obtained from GenBank or the *Parasteatoda* transcriptome (Posnien et al., 2014). Gene-specific cDNA fragments were amplified with primers designed with Primer3 (<http://primer3.ut.ee>) and cloned into pCR4-TOPO vector (Invitrogen, Life Technologies): *Pt-eve* (731 bp), GCAGGGTCTTCGAACTTCAG and GTTGGAAGAGTTGCGTCGTT; *Pt-cad* (1005 bp), TGTTGATGGGAGATGGTTCC and AAAGCCCCTTTCGAAGATGT; *Pt-cad* F1 (456bp) ATGTATTCCCCTACAGCTAGAC and ATCGCTGGAACTGCAACAATAG; *Pt-cad* F2 (429 bp)

GGTATGAGTGGTACTGAATCACC and
TCAGTAGATACTAATATTTGCTATATTTAGAG; *Pt-run-1* F1 (741 bp)
ATGCATTTACCAGCAGATTCAGTGA and
AACAGCGAGAGTGACATCCAAATTATA; *Pt-run-1* F2 (792 bp)
TCTCCAACATCTCAAGATTCATGTTC and TCAGTATGGCCTCCATAGACCT; *Pt-Dl*
(967 bp), ACAAACCACACGGCTTTTTTC and GCTTGGTCAAGCAGTCATCA; *Pt-N* F1
(701 bp) TGCAGCACATTCGAGACATG and CCGAGCCATTGTCTTCATCG; *Pt-N* F2
(675 bp) GTTCTCCTGGGCTAATGGGT and TCTTCTGGTGATGAGCTGCA; *Pt-Wnt8*
see McGregor et al., 2008.

RNA-probes were labeled with Digoxigenin (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, an additional probe was labeled with fluorescein (Roche) and detected with an alkaline phosphatase conjugated anti-fluorescein antibody (Fab fragments, Roche) and with INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)/BCIP (Roche), resulting in orange staining. *In situ* hybridisations were carried out according to the whole-mount protocol for spiders (Prpic et al., 2008) with minor modifications. The anti-DIG and anti-fluorescein antibodies were pre-absorbed overnight at 4°C with embryos from stages 6 to 8.2. 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 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 as for the regular *in situ* hybridization staining and then with the anti-Fluorescein

antibody at a dilution of 1:2000 in blocking solution 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. Segmental identity in stained embryos was assigned from morphological markers ascertained from images of DAPI stainings.

Double-stranded RNA preparation

Fragments of the coding regions of *Pt-Dl* (967 bp), *Pt-Wnt8* (718 bp), *Pt-cad* (1005 bp completely and as two non-overlapping fragments), *Pt-eve* (713 bp) *run-1* (672 bp as two non-overlapping fragments), *Pt-N* (1190 bp as two non-overlapping fragments) and GFP were amplified from plasmids using universal primers, which both contained a 5' T7 promoter binding site (Fwd T7 5'-TAATACGACTCACTATAGGG-3', Rev T7/T3 5'-TAATACGACTCACTATAGGGAATTAACCCTCACTAAAGGGA-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 with the MegaScript T7 transcription kit (Invitrogen). Double-stranded (ds) RNA was then generated by annealing the transcripts in a water bath starting at 95°C, and slowly cooled down to room temperature. The dsRNA was then adjusted to a concentration of 1.5 to 2.0 µg/µl for injections.

Parental RNAi (pRNAi)

For each gene, at least three adult female spiders were injected according to the protocol by Akiyama-Oda and Oda (2006). 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. Embryos from GFP injected control females were generated and treated as described above.

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). Embryos were injected at the 8- or 16-cell stage with an injection mix composed of 10 μ l Fluorescein isothiocyanate (FITC)-dextran (2 μ g/ μ l, MW 10 000, Sigma), 10ul Biotin-dextran (2 μ g/ μ l, MW 10 000, Sigma) and 5 μ l dsRNA (1.5 to 2.0 μ g/ μ l) and fixed when they reached the developmental stages 6 and 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.

Synthesis and overexpression of capped mRNA

An 885 bp fragment of the *Pt-cad* CDS was isolated and cloned using the pENTR™ Directional TOPO® Cloning Kit (Life Technologies®). The fragment was shuttled into pAWG, upstream of the enhanced GFP (eGFP), with the Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen®). The cad-eGFP construct was amplified from pAWG-cad-eGFP with 5' extensions containing *Pst*I and *Bam*HI for forward and reverse primers respectively. This construct was ligated into the pSP64 Poly(A) Vector (Promega) after double digest with *Pst*I and *Bam*HI. The pSP64-cad-eGFP-PolyA was then linearized with *Nhe*I and the resulting template was used for the SP6 transcription reaction with mMESSAGING mMACHINE® SP6 Transcription Kit (Ambion™) following the manufacturer's instructions. Capped eGFP-NLS mRNA was prepared from plasmid pSP64-NLS-tdEosFP-polyA-NotI+ (a gift from Hiroki

Oda and Yasuko Akiyama-Oda) as described previously (Kanayama et al., 2010). Capped mRNAs were injected as described by Kanayama et al., (2010).

Data documentation

Embryos were imaged using a Leica fluorescence stereomicroscope equipped with a Jenoptik ProgRes C3 digital camera. Bright field 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 (Fig S7) were taken with a Zeiss Axio Zoom V16 stereomicroscope, equipped with an AxioCam 506 mono and a colour digital camera.

Acknowledgements

This research was partially funded by an Oxford Brookes University Nigel Groome Studentship to AS and DJL, a CNPq scholarship to CLBP, EMBO short-term fellowship ASTF 304-2012 to MH and a Leverhulme visiting fellowship (VF-2012-016) to EES. We thank Hiroki Oda and Yasuko Akiyama-Oda for providing training on spider eRNAi experiments and providing us with plasmid pSP64-NLS-tdEosFP-polyA-NotI+. We thank Sebastian Kittelmann for comments on the manuscript and Christina Jahn for discussions about pair rule gene expression. We also thank the three reviewers for their very constructive comments and suggestions.

Author contributions statement

This study was designed and initiated by APM and WGMD. Experiments were carried out by AS assisted by MH, CLBP, DJL, EES and NMF. All authors contributed to analysis and

interpretation of the data. The manuscript was written by APM and AS with the help of all the other authors. All authors read and approved the manuscript.

References

- Akiyama-Oda, Y. and Oda, H.** (2003). Early patterning of the spider embryo: a cluster of mesenchymal cells at the cumulus produces Dpp signals received by germ disc epithelial cells. *Development* **130**, 1735-1747.
- Akiyama-Oda, Y. and Oda, H.** (2006). Axis specification in the spider embryo: dpp is required for radial-to-axial symmetry transformation and sog for ventral patterning. *Development* **133**, 2347-2357.
- Aranda, M., Marques-Souza, H., Bayer, T. and Tautz, D.** (2008). The role of the segmentation gene hairy in Tribolium. *Dev Genes Evol* **218**, 465-477.
- Bolognesi, R., Farzana, L., Fischer, T. D. and Brown, S. J.** (2008). Multiple Wnt genes are required for segmentation in the short-germ embryo of Tribolium castaneum. *Curr Biol* **18**, 1624-1629.
- Brena, C. and Akam, M.** (2013). An analysis of segmentation dynamics throughout embryogenesis in the centipede Strigamia maritima. *BMC Biol* **11**, 112.
- Brown, S. J., Parrish, J. K., Beeman, R. W. and Denell, R. E.** (1997). Molecular characterization and embryonic expression of the even-skipped ortholog of Tribolium castaneum. *Mech Dev* **61**, 165-173.
- Chesebro, J. E., Pueyo, J. I. and Couso, J. P.** (2013). Interplay between a Wnt-dependent organiser and the Notch segmentation clock regulates posterior development in Periplaneta americana. *Biology open* **2**, 227-237.
- Chipman, A. and Akam, M.** (2008). The segmentation cascade in the centipede Strigamia maritima: Involvement of the Notch pathway and pair-rule gene homologues. *Dev Biol* **319**, 160-169.
- Chipman, A. D., Arthur, W. and Akam, M.** (2004a). A double segment periodicity underlies segment generation in centipede development. *Curr Biol* **14**, 1250-1255.

- Chipman, A. D., Arthur, W. and Akam, M.** (2004b). Early development and segment formation in the centipede, *Strigamia maritima* (Geophilomorpha). *Evol Dev* **6**, 78-89.
- Choe, C. P., Miller, S. C. and Brown, S. J.** (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. *Proc Natl Acad Sci U S A* **103**, 6560-6564.
- Damen, W. G.** (2002). Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis. *Development* **129**, 1239-1250.
- Damen, W. G.** (2007). Evolutionary conservation and divergence of the segmentation process in arthropods. *Dev Dyn* **236**, 1379-1391.
- Damen, W. G., Weller, M. and Tautz, D.** (2000). Expression patterns of hairy, even-skipped, and runt in the spider *Cupiennius salei* imply that these genes were segmentation genes in a basal arthropod. *Proc Natl Acad Sci U S A* **97**, 4515-4519.
- Damen, W. G., Janssen, R. and Prpic, N. M.** (2005). Pair rule gene orthologs in spider segmentation. *Evol Dev* **7**, 618-628.
- Davis, G. K. and Patel, N. H.** (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annu Rev Entomol* **47**, 669-699.
- Davis, G. K., Jaramillo, C. A. and Patel, N. H.** (2001). Pax group III genes and the evolution of insect pair-rule patterning. *Development* **128**, 3445-3458.
- Dearden, P. K., Donly, C. and Grbic, M.** (2002). Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite *Tetranychus urticae*. In *Development*, vol. 129, pp. 5461-5472.
- El-Sherif, E., Zhu, X., Fu, J. and Brown, S. J.** (2014). Caudal regulates the spatiotemporal dynamics of pair-rule waves in *Tribolium*. *PLoS Genet* **10**, e1004677.

- Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M.** (1987). Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J* **6**, 749-759.
- Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A. A., Weir, M. and Goto, T.** (1996). *Drosophila* Paired regulates late even-skipped expression through a composite binding site for the paired domain and the homeodomain. *Development* **122**, 2697-2707.
- Green, J. and Akam, M.** (2013). Evolution of the pair rule gene network: Insights from a centipede. *Dev Biol* **382**, 235-245.
- Hilbrant, M., Damen, W. G. and McGregor, A. P.** (2012). Evolutionary crossroads in developmental biology: the spider *Parasteatoda tepidariorum*. *Development* **139**, 2655-2662.
- Hughes, C. L. and Kaufman, T. C.** (2002). Exploring myriapod segmentation: the expression patterns of even-skipped, engrailed, and wingless in a centipede. *Dev Biol* **247**, 47-61.
- Janssen, R., Budd, G. E., Prpic, N. M. and Damen, W. G.** (2011). Expression of myriapod pair rule gene orthologs. *EvoDevo* **2**, 5.
- Kageyama, R., Masamizu, Y. and Niwa, Y.** (2007). Oscillator mechanism of Notch pathway in the segmentation clock. *Dev Dyn* **236**, 1403-1409.
- Kanayama, M., Akiyama-Oda, Y. and Oda, H.** (2010). Early embryonic development in the spider *Achaearanea tepidariorum*: Microinjection verifies that cellularization is complete before the blastoderm stage. *Arthropod Struct Dev* **39**, 436-445.
- Kanayama, M., Akiyama-Oda, Y., Nishimura, O., Tarui, H., Agata, K. and Oda, H.** (2011). Travelling and splitting of a wave of hedgehog expression involved in spider-head segmentation. *Nat Commun* **2**, 500.
- McGregor, A. P.** (2006). Wasps, beetles and the beginning of the ends. *Bioessays* **28**, 683-686.

- McGregor, A. P., Pechmann, M., Schwager, E. E. and Damen, W. G.** (2009). An ancestral regulatory network for posterior development in arthropods. *Commun Integr Biol* **2**, 174-176.
- McGregor, A. P., Hilbrant, M., Pechmann, M., Schwager, E. E., Prpic, N. M. and Damen, W. G.** (2008a). *Cupiennius salei* and *Achaearanea tepidariorum*: Spider models for investigating evolution and development. *Bioessays* **30**, 487-498.
- McGregor, A. P., Pechmann, M., Schwager, E. E., Feitosa, N. M., Kruck, S., Aranda, M. and Damen, W. G.** (2008b). Wnt8 is required for growth-zone establishment and development of opisthosomal segments in a spider. *Curr Biol* **18**, 1619-1623.
- Mito, T., Kobayashi, C., Sarashina, I., Zhang, H., Shinahara, W., Miyawaki, K., Shinmyo, Y., Ohuchi, H. and Noji, S.** (2007). *even-skipped* has gap-like, pair-rule-like, and segmental functions in the cricket *Gryllus bimaculatus*, a basal, intermediate germ insect (Orthoptera). *Dev Biol* **303**, 202-213.
- Mittmann, B. and Wolff, C.** (2012). Embryonic development and staging of the cobweb spider *Parasteatoda tepidariorum* C. L. Koch, 1841 (syn.: *Achaearanea tepidariorum*; Araneomorphae; Theridiidae). *Dev Genes Evol* **222**, 189-216.
- Oberhofer, G., Grossmann, D., Siemanowski, J. L., Beissbarth, T. and Bucher, G.** (2014). Wnt/beta-catenin signaling integrates patterning and metabolism of the insect growth zone. *Development* **141**, 4740-4750.
- Oda, H., Nishimura, O., Hirao, Y., Tarui, H., Agata, K. and Akiyama-Oda, Y.** (2007). Progressive activation of Delta-Notch signaling from around the blastopore is required to set up a functional caudal lobe in the spider *Achaearanea tepidariorum*. *Development* **134**, 2195-2205.
- Pankratz, M. and Jäckle, H.** (1993). Blastoderm segmentation. In *The development of Drosophila melanogaster* (eds M. Bate and A. Martinez Arias), pp. 467-516: Cold Spring Harbor Press.

- Patel, N. H., Condrón, B. G. and Zinn, K.** (1994). Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. *Nature* **367**, 429-434.
- Pechmann, M., McGregor, A. P., Schwager, E. E., Feitosa, N. M. and Damen, W. G.** (2009). Dynamic gene expression is required for anterior regionalization in a spider. *Proc Natl Acad Sci U S A* **106**, 1468-1472.
- Pechmann, M., Khadjeh, S., Turetzek, N., McGregor, A. P., Damen, W. G. and Prpic, N. M.** (2011). Novel function of Distal-less as a gap gene during spider segmentation. *PLoS Genet* **7**, e1002342.
- Peel, A. D., Chipman, A. D. and Akam, M.** (2005). Arthropod segmentation: beyond the *Drosophila* paradigm. *Nat Rev Genet* **6**, 905-916.
- Posnien, N., Zeng, V., Schwager, E. E., Pechmann, M., Hilbrant, M., Keefe, J. D., Damen, W. G. M., Prpic, N.-M., McGregor, A. P. and Extavour, C. G.** (2014). A Comprehensive Reference Transcriptome Resource for the Common House Spider *Parasteatoda tepidariorum*. *PLoS ONE* **9**, e104885.
- Prpic, N. M., Schoppmeier, M. and Damen, W. G.** (2008). Whole-mount in situ hybridization of spider embryos. *CSH Protoc* **2008**, pdb.prot5068.
- Pueyo, J. I., Lanfear, R. and Couso, J. P.** (2008). Ancestral Notch-mediated segmentation revealed in the cockroach *Periplaneta americana*. *Proc Natl Acad Sci U S A* **105**, 16614-16619.
- Rosenberg, M. I., Brent, A. E., Payre, F. and Desplan, C.** (2014). Dual mode of embryonic development is highlighted by expression and function of *Nasonia* pair-rule genes. *eLife* **3**, e01440.
- Sarrazin, A. F., Peel, A. D. and Averof, M.** (2012). A segmentation clock with two-segment periodicity in insects. *Science* **336**, 338-341.

- Schoppmeier, M. and Damen, W. G.** (2005a). Suppressor of Hairless and Presenilin phenotypes imply involvement of canonical Notch-signalling in segmentation of the spider *Cupiennius salei*. *Dev Biol* **280**, 211-224.
- Schoppmeier, M. and Damen, W. G.** (2005b). Expression of Pax group III genes suggests a single-segmental periodicity for opisthosomal segment patterning in the spider *Cupiennius salei*. *Evol Dev* **7**, 160-169.
- Schwager, E. E., Pechmann, M., Feitosa, N. M., McGregor, A. P. and Damen, W. G.** (2009). hunchback functions as a segmentation gene in the spider *Achaearanea tepidariorum*. *Curr Biol* **19**, 1333-1340.
- Schwager, E. E., Schoenauer, A., Leite, D. J., Sharma, P. P. and McGregor, A. P.** (2015). Chelicerata. In *Evolutionary Developmental Biology of Invertebrates* (ed. A. Wanninger). Berlin: Springer.
- Shinmyo, Y., Mito, T., Matsushita, T., Sarashina, I., Miyawaki, K., Ohuchi, H. and Noji, S.** (2005). caudal is required for gnathal and thoracic patterning and for posterior elongation in the intermediate-germband cricket *Gryllus bimaculatus*. *Mech Dev* **122**, 231-239.
- Small, S., Blair, A. and Levine, M.** (1992). Regulation of even-skipped stripe 2 in the *Drosophila* embryo. *EMBO J* **11**, 4047-4057.
- Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M.** (1991). Transcriptional regulation of a pair-rule stripe in *Drosophila*. *Genes Dev* **5**, 827-839.
- Sommer, R. J. and Tautz, D.** (1993). Involvement of an orthologue of the *Drosophila* pair-rule gene hairy in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* **361**, 448-450.
- Stollewerk, A., Schoppmeier, M. and Damen, W. G.** (2003). Involvement of Notch and Delta genes in spider segmentation. *Nature* **423**, 863-865.
- Tautz, D.** (2004). Segmentation. *Dev Cell* **7**, 301-312.

Figure legends

Figure 1. *Pt-Dl* regulates *Pt-Wnt8* expression

In wild-type embryos at stage 6, *Pt-Dl* is expressed in a salt and pepper pattern adjacent to a more diffuse stripe (indicated by dashed lines) in anterior SAZ cells (**A**). At stage 6, *Pt-Wnt8* is strongly expressed in posterior SAZ cells (**B**). Expression of *Pt-Wnt8* is comparatively weaker in anterior SAZ cells where *Pt-Dl* is expressed at this stage. In *Pt-Dl* pRNAi embryos at stage 6, *Pt-Wnt8* expression is lost in posterior SAZ cells but this gene is conversely expressed more strongly in anterior SAZ cells compared to wild-type (**C**). Images **A'-C'** show the fluorescent staining with the nuclear marker DAPI of the respective bright field images **A-C**. All panels show posterior views of whole mount embryos, ventral is oriented to the left. Abbreviations: wt, wild-type. Developmental stages are indicated.

Figure 2. Dynamic *Pt-eve* expression during early embryogenesis in *Parasteatoda*

At mid stage 6, *Pt-eve* is expressed in a small circular domain in the SAZ (**A**). Expression then clears from the centre (indicated by a black arrow) at a slightly later stage (**B**), until it disappears entirely from posterior SAZ cells leaving a crescent shaped expression domain (**C**). Subsequently, *Pt-eve* is expressed in the forming O1 segment in anterior SAZ cells and a new domain forms in posterior SAZ cells (**D**), from which it fades again centrally at mid stage 7 (**E**). At late stage 7, the SAZ is clear of *Pt-eve* expression, but now the gene is expressed strongly in the posterior portion of the forming O2 segment and comparably more weakly in the O1 segment (**F**). At stage 8.1, a new domain of *Pt-eve* expression is observed in the SAZ, while expression is still observed in O2 and O3 (**G**). *Pt-eve* expression is observed in the SAZ (marked with an asterisk) and also in the forming nervous system at stage 9.1 (indicated by an arrow) (**H**). All images show whole mount embryos with anterior to the left as overlays of the

bright field channel depicting the expression pattern and a fluorescent channel showing DAPI nuclear staining in blue. Abbreviations: O, opisthosomal segment.

Figure 3. Effects of *Pt-Dl* and *Pt-Wnt8* pRNAi on *Pt-eve*

Expression of *Pt-eve* in wild-type (A), *Pt-Delta* pRNAi (B) and *Pt-Wnt8* pRNAi (C) embryos. 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). (A-C) whole mount embryos showing the posterior end of the opisthosoma with anterior to the left. The dashed circle in B indicates the posterior region of the SAZ A. Panels A'-C' show the DAPI staining of the respective bright field/DAPI overlay images A-C. Abbreviations as in Figure 1.

Figure 4. *Pt-eve* and *Pt-cad* exhibit overlapping expression and *Pt-cad* regulates *Pt-eve*

At mid stage 6, *Pt-eve* (blue) and *Pt-cad* (orange) expression largely overlap in the SAZ (A) but *Pt-cad* expression remains in cells where *Pt-eve* expression has cleared (white arrows in A'). *Pt-eve* and *Pt-cad* expression also overlap at a later stage (C) in two to three cell rows within the forming segment (white arrows in C'). Although *Pt-eve* alone is expressed in a single row of anterior cells (black arrows), *Pt-cad* is also expressed in two to three cells posteriorly to *Pt-eve* expressing cells (black asterisks in C'). *Pt-eve* expression is strongly down-regulated in the *Pt-cad* eRNAi clones (red) in the SAZ (B, and indicated by a black arrow in D) and at a later stage in the SAZ including the forming O1 segment (D). In all panels anterior is oriented to the left and all embryos are counterstained with DAPI. A, C whole mount embryos in a posterior and ventral view of the germ disc and germ band respectively with *Pt-eve* expression in blue and *Pt-cad* in orange. A' and C' show a higher magnification of the embryos in A and C respectively. Abbreviations as in Figure 1.

Figure 5. *Pt-eve* does not regulate *Pt-run-1*

Pt-eve and *Pt-run-1* are expressed in overlapping circular and crescent shaped domains respectively in the anterior and posterior of the SAZ (A). *Pt-run-1* expression appears normal in *Pt-eve* eRNAi knockdown cell clones (red) (B). Both panels show flat mounts of the posterior region of the germ band of mid stage 7 embryos, with anterior to the left. Abbreviations as in Figure 1.

Figure 6. Regulatory interactions in the *Parasteatoda* SAZ

In the posterior cells of the SAZ, *Dl-N* activates *Wnt8* expression to maintain a pool of undifferentiated cells. *Wnt8* is then required for dynamic expression of *Dl* that results in the formation of a stripe of *Dl* expression in anterior SAZ cells. *Wnt8* and *Dl-N* are also required to activate *cad* expression, and together these factors activate *eve* and *runt* expression. In anterior SAZ cells, *Dl* then suppresses *Wnt8* expression and in combination with *cad*, *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 if additional factors are required. Note that for simplicity we have only illustrated the regulation of *Wnt8* by *Dl* and *N* rather than the regulation of *Dl* and *N* expression on each other.

Supplementary figures

Figure S1. *Pt-cad* eRNAi does not affect *Pt-Dl* expression

In wild-type embryos at stage 7, *Pt-Dl* (blue) and *Pt-cad* (orange) are expressed in distinct but overlapping domains (indicated by chevrons and arrowheads in anterior and posterior cells respectively) in the SAZ (A). In *Pt-cad* eRNAi knockdown cell clones (red), *Pt-Dl* expression

(blue) appears to be unaffected. Flat mounted embryos in **A** and **B** are oriented with their anterior to the left.

Figure S2. Expression and regulation of *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. Abbreviations: wt, wild-type. Developmental stages are indicated.

Figure S3. *Pt-N* regulates *Pt-Wnt8* expression in the SAZ of *Parasteatoda*

In wild-type embryos at stage 7, *Pt-Wnt8* is expressed in the posterior SAZ cells and more weakly in the anterior SAZ cells (**A**). Expression of *Pt-Wnt8* is lost in the posterior, but is expressed more highly in the anterior SAZ in *Pt-N* parental RNAi embryos at that stage (**B**). Images **A'-B'**; show the fluorescent staining with the nuclear marker DAPI of the respective bright field images **A, B**. All panels show posterior views of whole mount embryos with ventral oriented to the left. Abbreviations: wt, wild-type. Developmental stages are indicated.

Figure S4. *Pt-cad* expression during *Parasteatoda* embryogenesis

At mid stage 6, *Pt-cad* is expressed in a circular domain in the SAZ (**A**), from which it then clears centrally as it expands (**B**). Subsequently, *Pt-cad* continues to be expressed in a broad

anterior crescent shaped domain and a posterior circular domain (C). At late stage 7, *Pt-cad* expression appears in the prosoma in a 1 to 2 cell wide stripe (indicated by a black arrow) and is also strongly expressed in the anterior portion of O1 segment and throughout the SAZ (D). The expression in the prosoma (black arrow) then broadens slightly, whereas expression in the forming O1 segment fades, and expression is observed in anterior and posterior SAZ cells (E). At stage 8.2, *Pt-cad* is expressed strongly in the SAZ, shows faint expression in O2 and strong prosomal expression in a 4-5 cells wide stripe (black arrow) (F). In all panels anterior is to the left and embryos are counterstained with DAPI. Whole mount embryos with a ventral view of stage 6 embryos (A, B) and opisthosomal part of the germ band (C-F), respectively. Abbreviations: L, walking limb; O, opisthosomal segment; wt, wild-type. Developmental stages are indicated.

Figure S5. Embryonic RNAi controls and *Pt-eve* knockdown does not affect *Pt-cad* expression

Expression of *Pt-cad* and *Pt-eve* is strongly down regulated in the *Pt-cad* and *Pt-eve* eRNAi cell clones (red) (outlined with white dashed lines), respectively (A, B). Expression of *Pt-cad* in the SAZ (C) and in the forming O1 segment (D) appears unaffected in *Pt-eve* eRNAi cell clones. In all panels anterior is to the left and embryos in C, D are counterstained with DAPI. Flat mount embryos in a ventral view of a stage 6 embryo (A, C) and opisthosomal part of the germ band (B, D), respectively.

Figure S6. *Pt-eve* and *Pt-cad* expression

Pt-cad (orange) and *Pt-eve* (blue) are initially co-expressed in about 20 cells in the SAZ (A), although *Pt-cad* appears to be expressed first (indicated by arrows in A'). Expression of both genes then clears from the posterior and *Pt-cad* and *Pt-eve* are expressed in an overlapping

crescent shaped domain in which cells in the anterior and posterior express only *Pt-eve* or *Pt-cad* respectively (**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. *Pt-cad* expression is broader than that of *Pt-eve* and persists for longer in the SAZ (**D, E**). In all panels anterior is to the left and embryos are counterstained with DAPI. Whole mount embryos in a ventral view of stage 6 embryos (**A-C**) and opisthosomal germ band (**D, E**), respectively. Abbreviations as in Figure S1.

Figure S7. *Pt-cad* expression is not sufficient to induce *Pt-eve* expression

The injected embryo shown was imaged at stage 5, when the primary thickening (Pt) can be found in the center of the germ disc (**A**). Injection with *Pt-cad::eGFP/RITC-dextran* (at the 16 cell stage) generated a clone of cells which exhibit specific nuclear eGFP expression and exclusively cytoplasmic signal of the fluorescent marker Rhodamine B (RodB) (**B**). After in situ hybridisation, *Pt-eve* expression could not be detected in any cells of the respective *Pt-cad::eGFP/RITC-dextran* injected embryo (**C**). Panels **D-F** show a higher magnification of the same *Pt-cad::eGFP/RITC-dextran* clone, whereby RITC-dextran is only detected in the cytoplasm (**E**) and eGFP is expressed in the nuclei of the clone cells (**F**). Panel **A** shows a bright field image of the injected embryo. Panels **B, D-F** show images of the same live embryo. Panel **C** shows the same embryo after fixation and *Pt-eve* in situ hybridisation, overlaid with a DAPI counterstain image. Abbreviation: Pt, primary thickening.

Figure S8. *Pt-run-1* expression during *Parasteatoda* embryogenesis

In situ hybridisation of *Pt-run-1* (**A-F**) and double in situ hybridisation of *Pt-run-1* and *Pt-eve* (**G-I**). In all panels anterior is to the left and embryos in **A-F** are counterstained with DAPI. Whole mount embryos with a ventral view of stage 6 embryos (**A-C**) and the opisthosomal

region of the germ band (**D-F**), respectively. **G-I** show flat mounted embryos with double in situ stainings, where *Pt-run-1* expression is shown in blue and *Pt-eve* in orange. *Pt-run-1* expression commences in a circular domain in the SAZ at stage 6 (**A**). This domain develops into a ring shaped domain (**B**) and new expression is then observed in posterior SAZ cells (**C**). *Pt-run-1* expression is limited to an anterior stripe in the SAZ and faint expression in O1 at stage 7 (**D**). Expression in O1 subsequently becomes stronger and the expression domain in anterior SAZ cells narrows (**E**). During stage 8.1, *Pt-run-1* continues to be expressed in O1 and appears again in the SAZ and also in the anterior region of O2 (**F**). *Pt-run-1* and *Pt-eve* expression largely overlap during posterior development, although *Pt-eve* is expressed approximately three cell rows anterior to *Pt-run-1* (indicated by black arrows in **G-I**). Abbreviations as in Figure S1.

Figure S9. *Pt-run-1* is not required for the activation of *Pt-eve*

The embryonic knockdown of *Pt-run-1* was tested with two non-overlapping fragments (F1, F2) and led to the loss of *Pt-run-1* expression in the clone area for both fragments (**A, B**).

The knockdown of *Pt-run-1* (only F2 is shown) had no effect on the anterior stripe expression (**C**) or the SAZ expression of *Pt-eve* (**D**). Note that embryo in C has been damaged in the SAZ area upon flat mounting, the missing *Pt-eve* expression in the posterior SAZ is a technical artifact. All panels show flat mount embryos with the anterior oriented to the left. Abbreviations: wt, wild-type. Developmental stages are indicated.

Figure S10. Effects of *Pt-Dl*, *Pt-Wnt8* pRNAi and *Pt-cad* eRNAi on *Pt-run-1* expression

Expression of *Pt-run-1* in wild-type (**A**), *Pt-Dl* pRNAi (**B**) and *Pt-Wnt8* pRNAi (**C**) embryos. In *Pt-Dl* pRNAi embryos, *Pt-run-1* expression is no longer detectable (**B**), compared to a wild-type *Pt-run-1* expression (**A**). Note that the dense accumulations of cells in the posterior

of the *Pt-Dl* pRNAi phenotype embryo (B), causes strong background signal in the DAPI staining. In *Pt-Wnt8* pRNAi embryos, expression of *Pt-run-1* is reduced to only a few cells (C). Embryonic *Pt-cad* RNAi results in a loss of expression of *Pt-run-1* in the SAZ (D). (A-C) whole mount embryos and D is a flat mount embryo, showing the posterior end of the opisthosoma with anterior to the left. Panels A'-D' show the DAPI staining of the respective bright field/DAPI overlay images A-D. Abbreviations as in Figure S1.

Figure 1

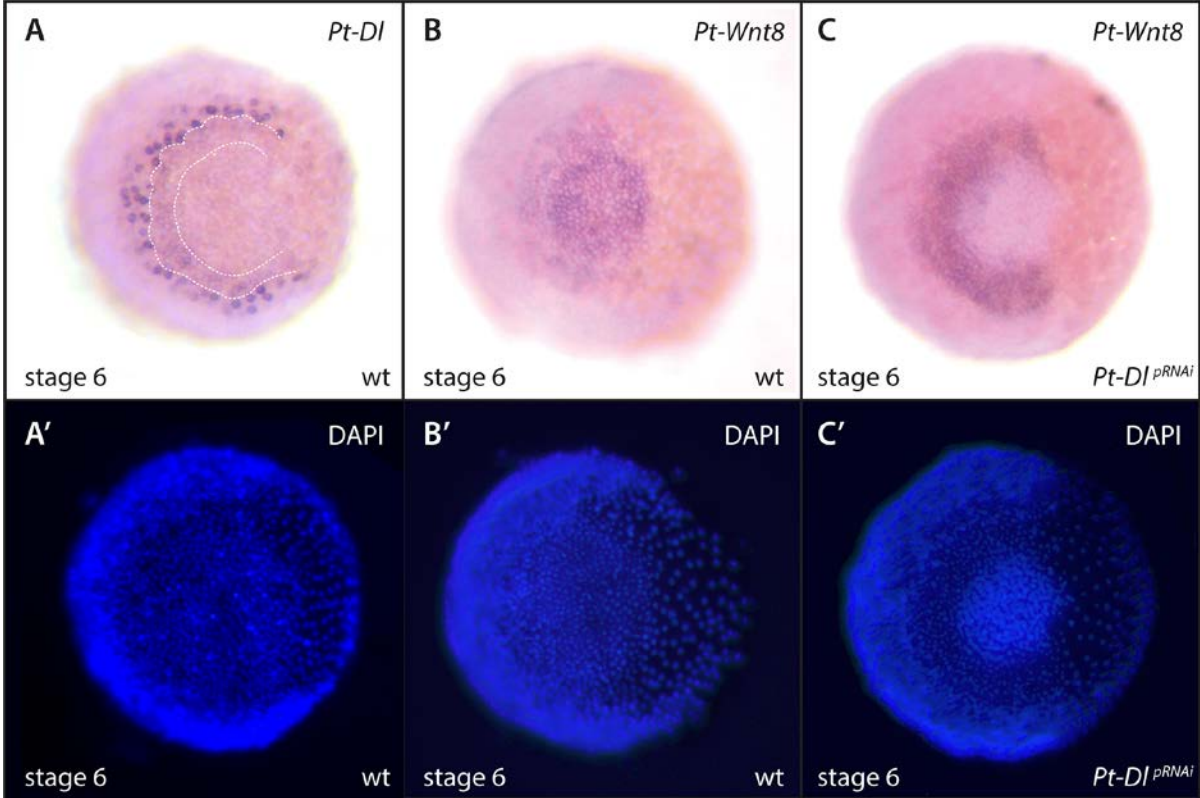


Figure 2

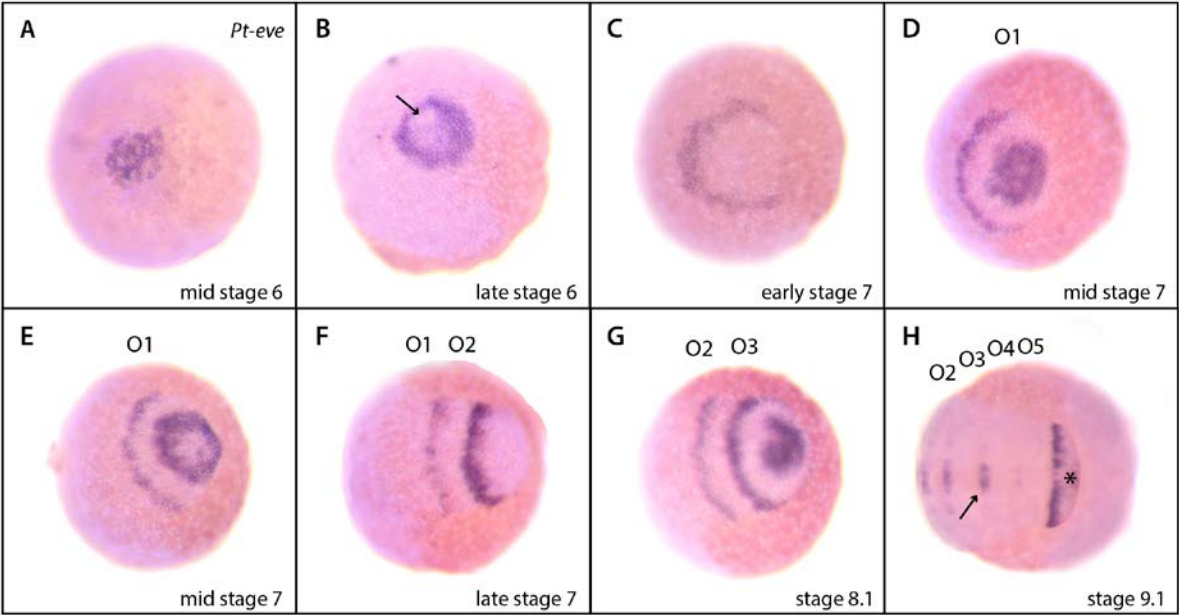


Figure 3

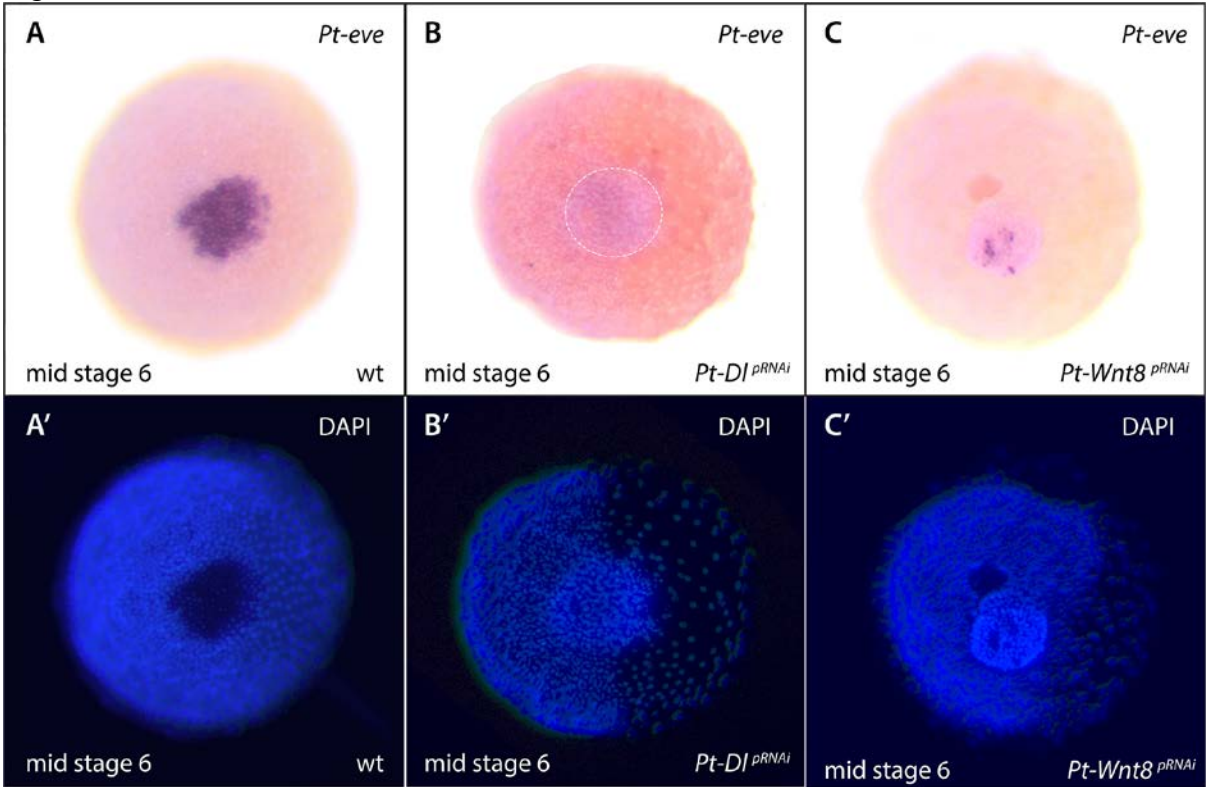


Figure 4

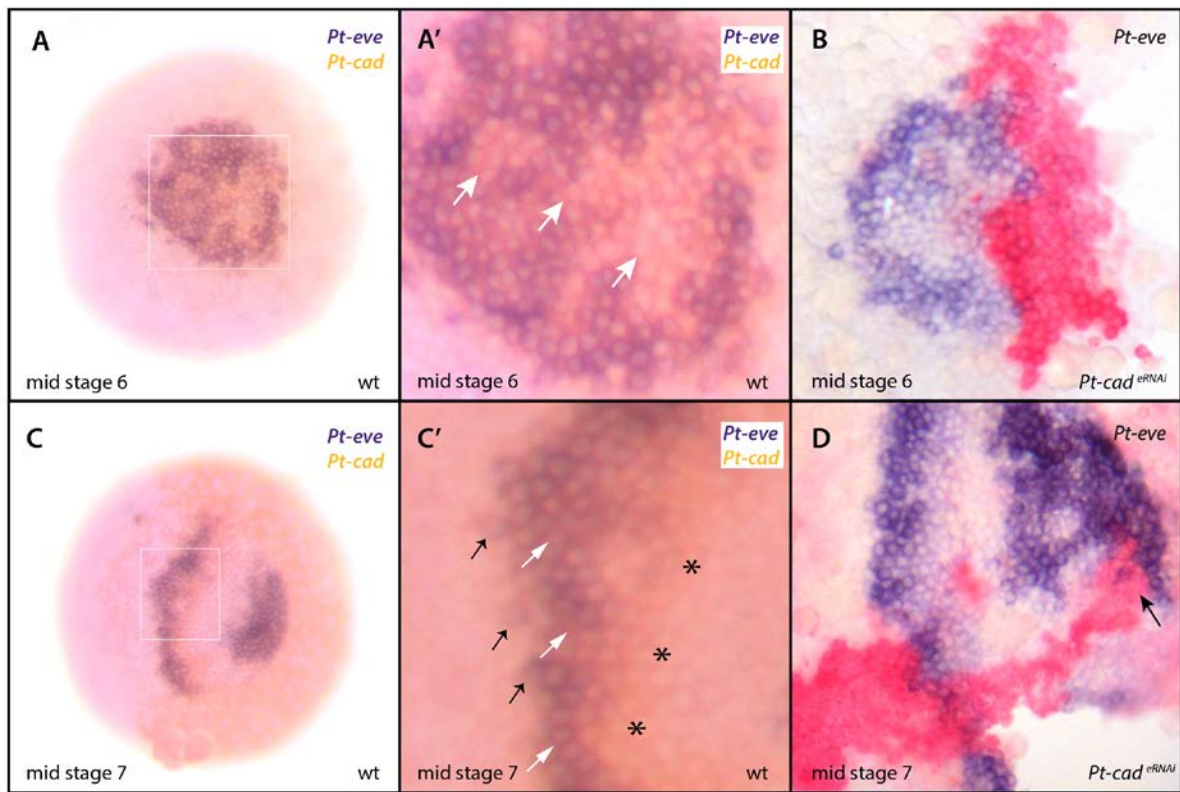


Figure 5

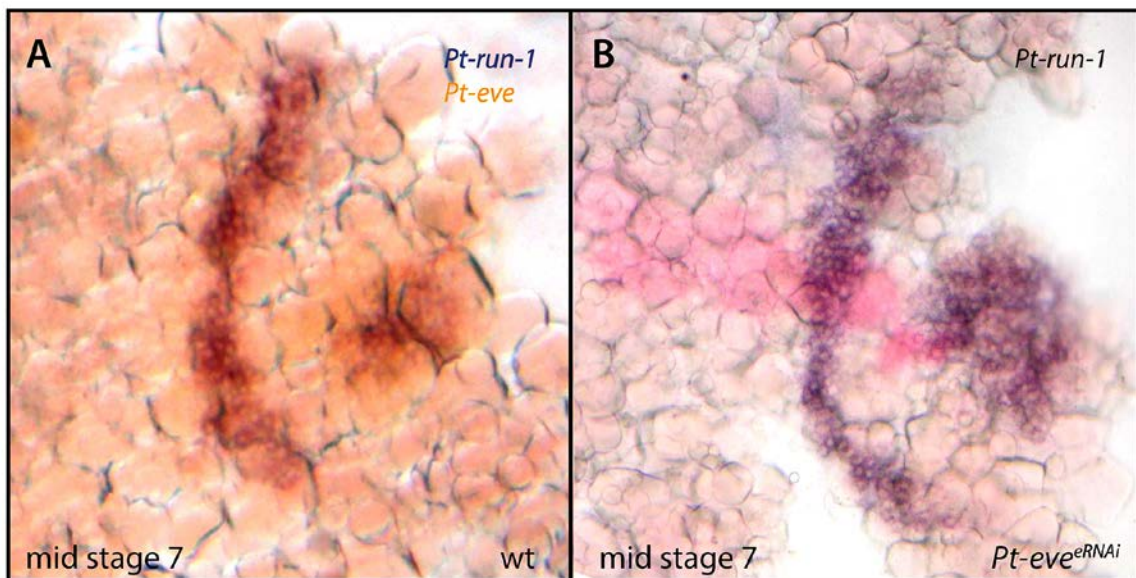
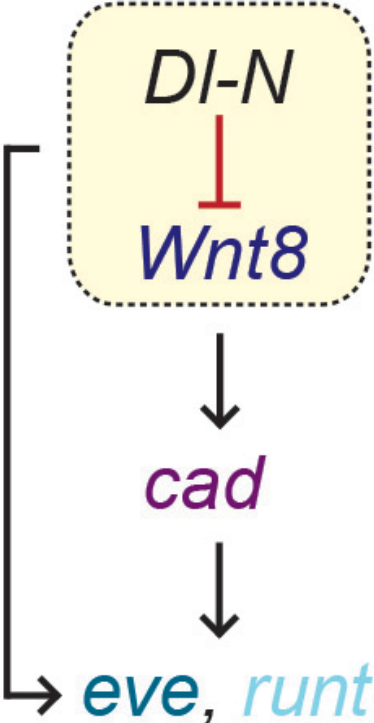


Figure 6

Anterior SAZ



Posterior SAZ

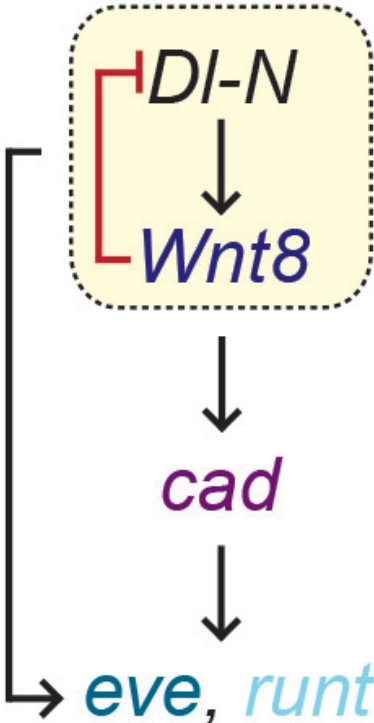


Figure S1

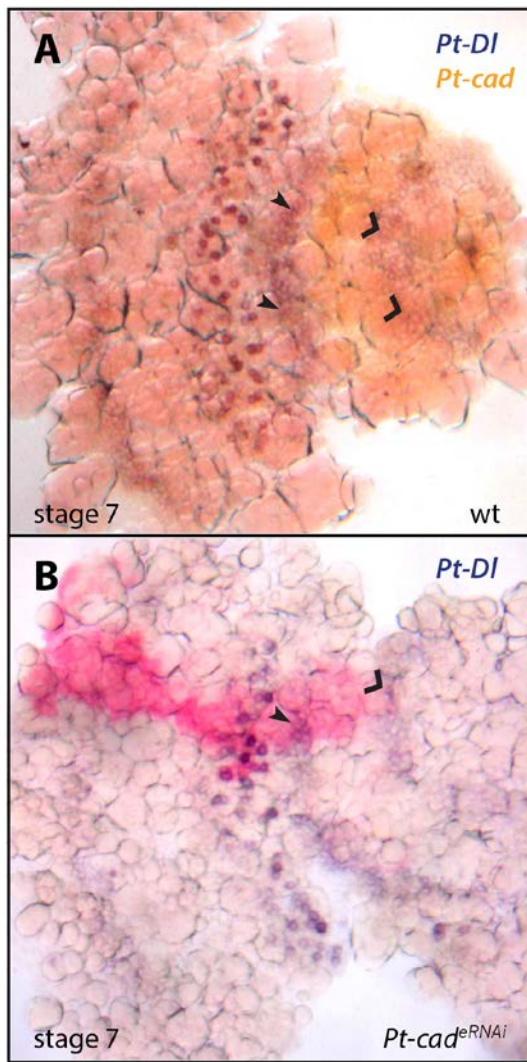


Figure S2

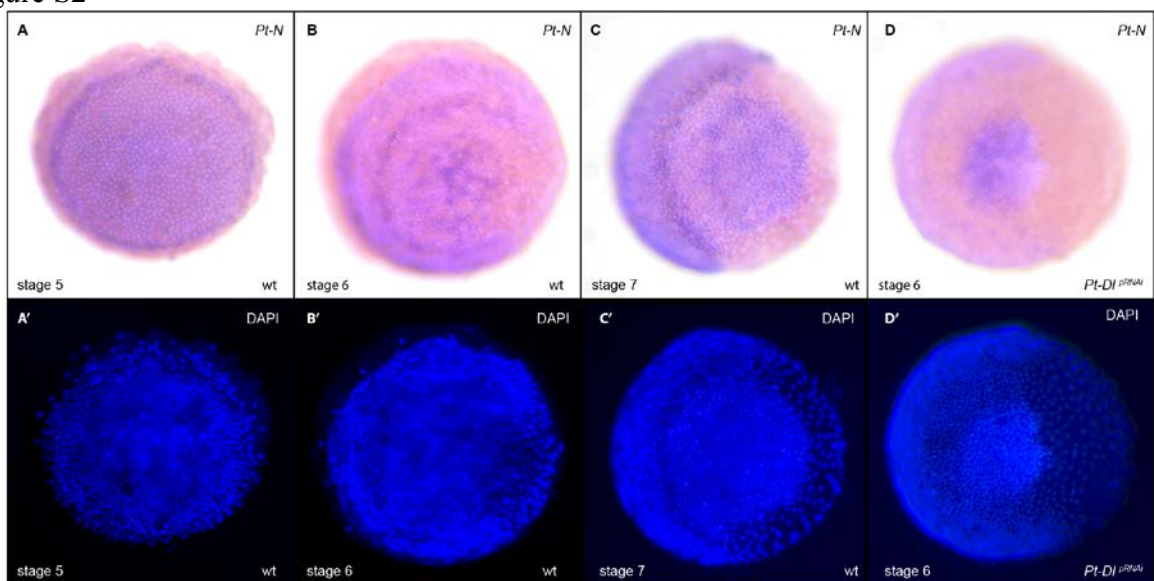


Figure S3

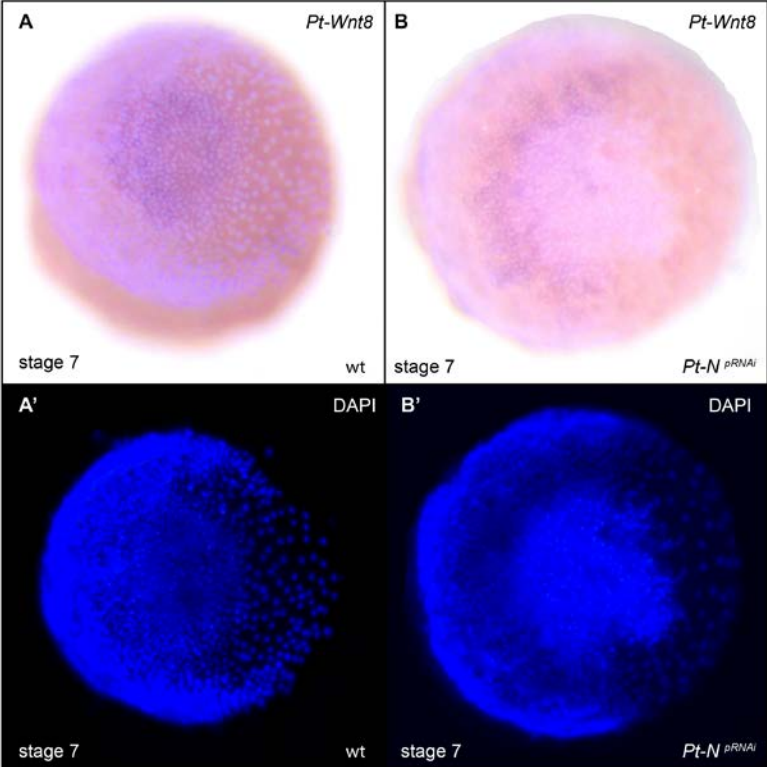


Figure S4

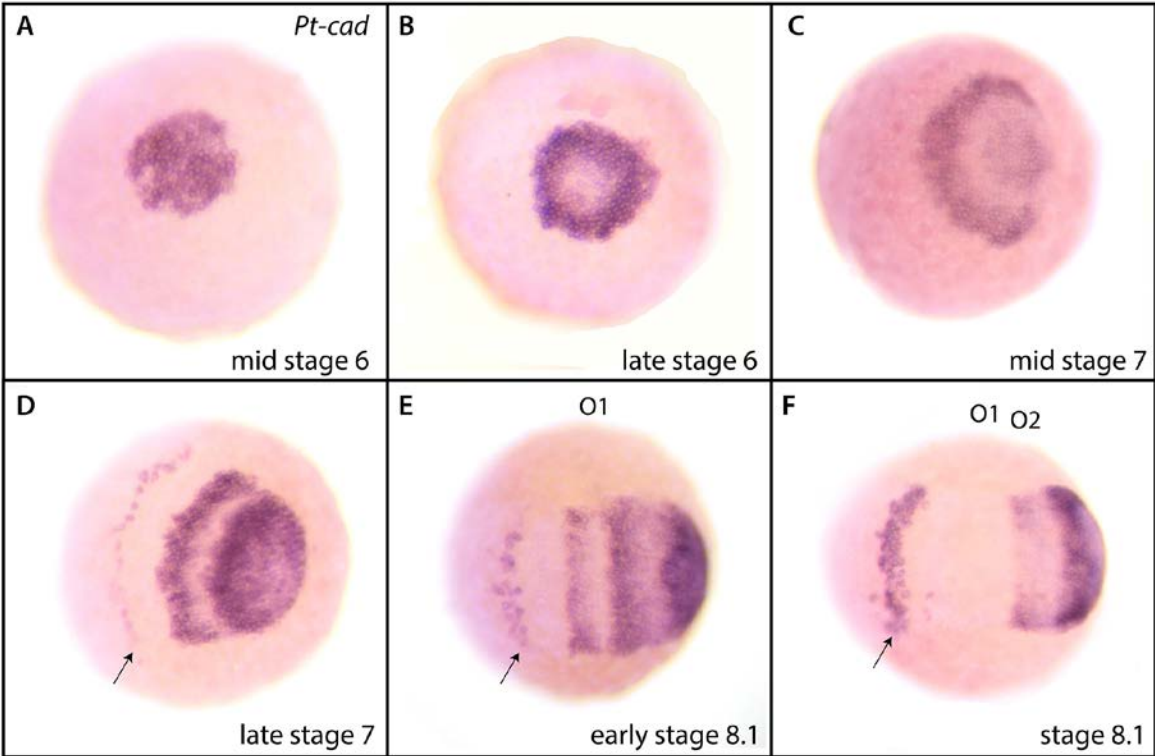


Figure S5

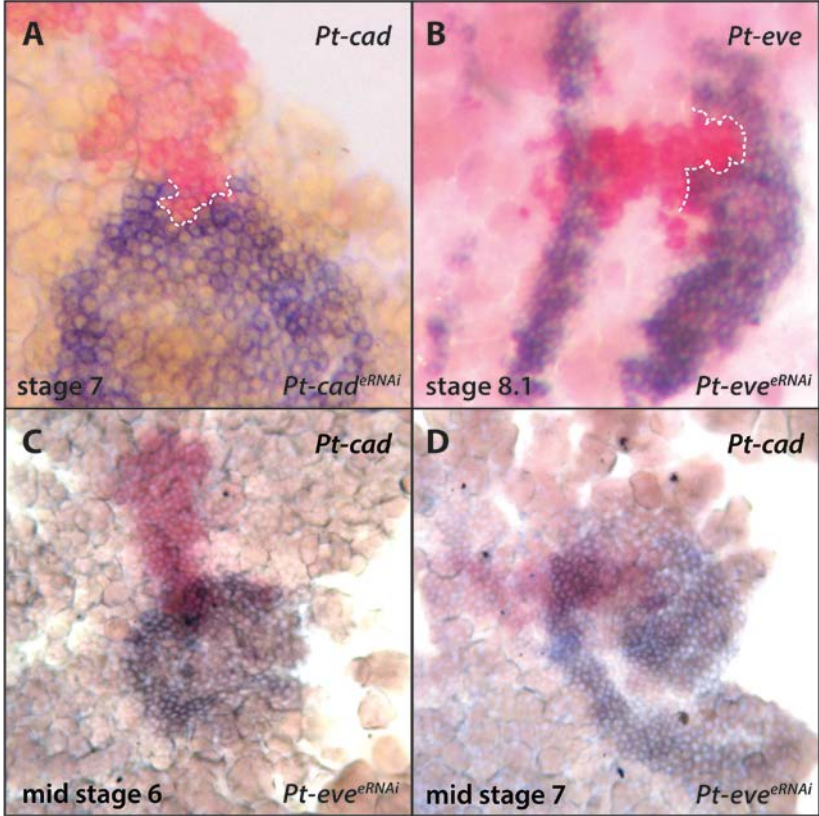


Figure S6

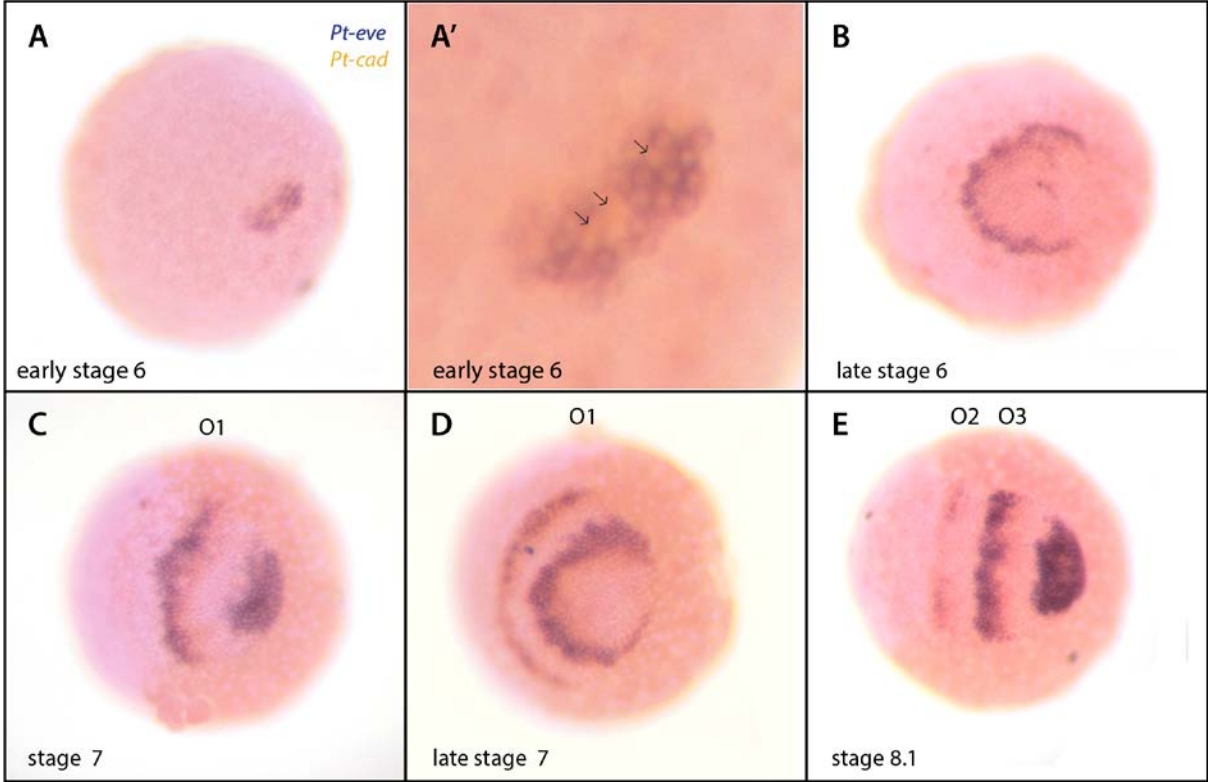


Figure S7

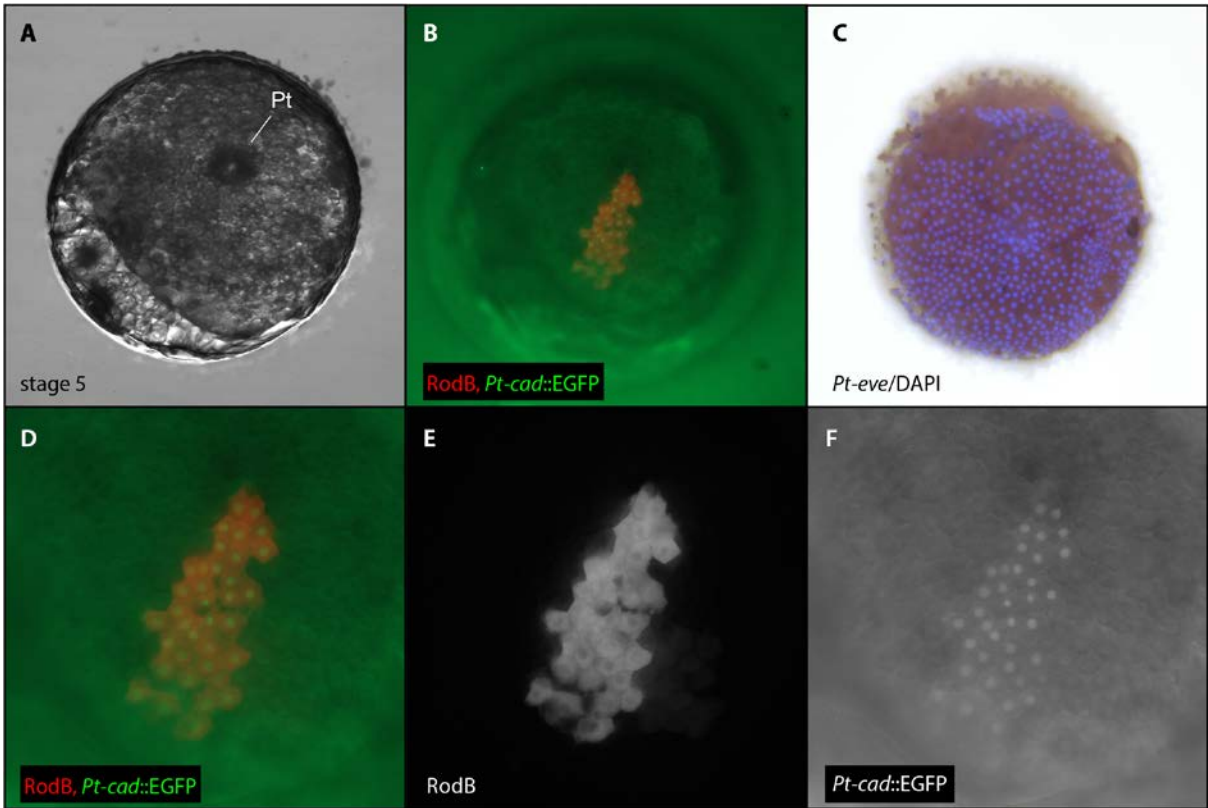


Figure S8

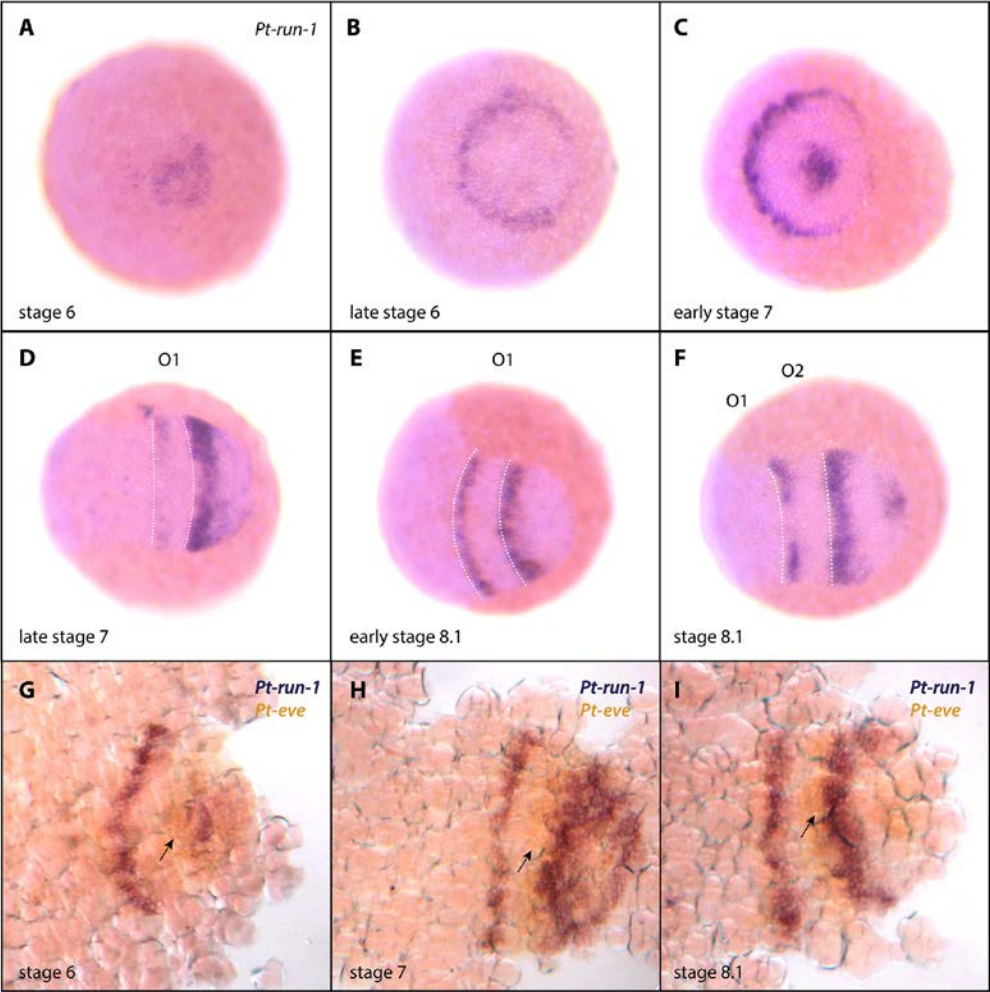


Figure S9

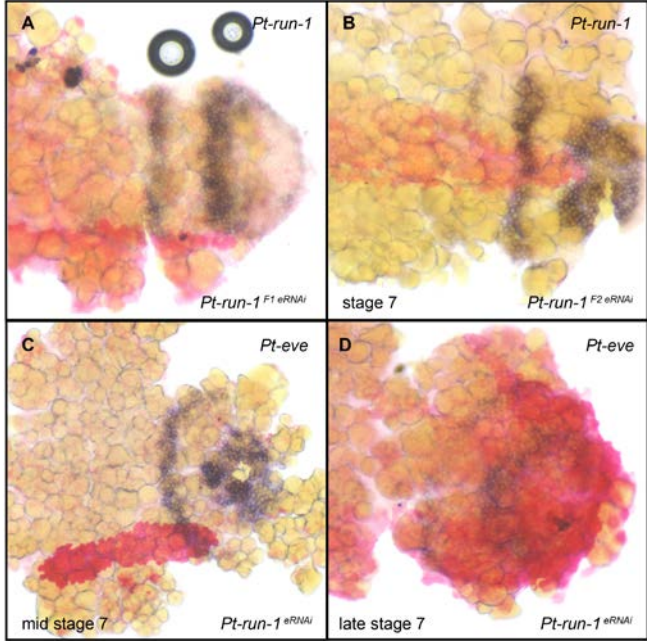


Figure S10

