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Genetic regulation of *engrailed* and *wingless* in *Tribolium* segmentation and the evolution of pair-rule segmentation

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ABSTRACT

In *Drosophila*, primary pair-rule genes establish the parasegmental boundaries and indirectly control the periodic expression of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) via regulation of secondary pair-rule genes. Although orthologs of some *Drosophila* pair-rule genes are not required for proper segmentation in *Tribolium*, segmental expression of *Tc-en* and *Tc-wg* is conserved. To understand how these segment polarity genes are regulated, we examined the results of expressing one or two pair-rule genes in the absence of the other known pair-rule genes. Expression of one or both of the secondary pair-rule genes, *Tc-sloppy-paired* (*Tc-slp*) and *Tc-paired* (*Tc-prd*), activated *Tc-wg* in the absence of the primary pair-rule genes, *Tc-even-skipped* (*Tc-eve*), *Tc-runt* (*Tc-run*) and *Tc-odd-skipped* (*Tc-odd*). *Tc-eve* alone failed to activate *Tc-wg* or *Tc-en*, but in combination with *Tc-run* or *Tc-prd* activated *Tc-en*. These results, interpreted within the pair-rule gene expression patterns, suggest separate models for the genetic regulation of the juxtaposed expression of *Tc-wg* and *Tc-en* at odd- and even-numbered parasegmental boundaries, respectively. Conserved interactions between *eve* and *prd* at the anterior boundary of odd-numbered parasegments may reflect an ancestral segmentation mechanism that functioned in every segment prior to the evolution of pair-rule segmentation.

Introduction

Segmentation, a defining character of arthropods, is best understood in the long-germ insect, *Drosophila melanogaster*. Detailed genetic and molecular analysis has revealed a well-organized segmentation hierarchy of maternal, gap, pair-rule and segment polarity genes that subdivide the embryo along the anterior–posterior axis into narrow regions and finally into repeated segments (recently reviewed by Peel et al., 2005). Primary pair-rule genes, which are regulated by maternal and gap genes, define parasegmental boundaries whereas secondary pair-rule genes, which are mainly regulated by primary pair-rule genes, directly regulate segment polarity genes to pattern segments. Even though *Drosophila* is considered to be an evolutionarily derived species, the genetic and molecular mechanisms of segmentation found in *Drosophila* provide a model system for comparative studies to understand the evolution of segmentation mechanisms in short-germ insects and other arthropods, where segmentation occurs progressively from anterior to posterior. Most notably, the juxtaposed expression of *wingless* (*wg*) and *engrailed* (*en*), required for proper segmental boundary formation in *Drosophila*, is highly conserved (Angelini and Kaufman, 2005; Brown et al., 1994b; Damen, 2002; Hughes and Kaufman, 2002; Kettle et al., 2003; Manzanares et al., 1993; Nagy and Carroll, 1994; Peterson et al., 1998; Sommer and Tautz, 1991 and Fig. 1B). Parasegments, genetically regulated development units delimited by *en* and *wg* expression, appear to be functionally conserved among arthropods (Damen, 2002). Furthermore, orthologs of pair-rule genes are expressed in repeating patterns in diverse arthropods (for review see Damen, 2007; Peel et al., 2005).

Accumulating genetic and molecular evidence from other insects and arthropods suggest that both the functions of pair-rule gene orthologs and the interactions by which they regulate segment polarity genes have diverged considerably. Certain pair-rule genes such as *even-skipped* (*eve*), *runt* (*run*), *hairy* (*h*) or *fushi tarazu* (*ftz*) are activated in patterns of double segment periodicity in *Drosophila* and other holometabolous insects including *Anopheles gambia* (Goltsev et al., 2004), *Apis melifera* (Binner and Sander, 1997), *Bombyx mori* (Xu et al., 1997), *Manduca sexta* (Kraft and Jackle, 1994) and *Tribolium castaneum* (Brown et al., 1994a; Patel et al., 1994; Sommer and Tautz, 1993). In several of these insects, expression of pair-rule genes resolves into secondary, segmentally-reiterated patterns. While in still other insects (Grbic et al., 1996) and arthropod groups including myriapods (Hughes and Kaufman, 2002), chelicerates (Damen et al., 2000) and crustaceans (Copf et al., 2003) they are expressed in segmental rather than pair-rule patterns. On the other hand, pair-rule genes such as *paired* (*prd*) or *odd-skipped* (*odd*) are expressed in double-segmental patterns in at least one spider and a centipede, respectively (Chipman et al., 2004; Dearden et al., 2002), as well as in

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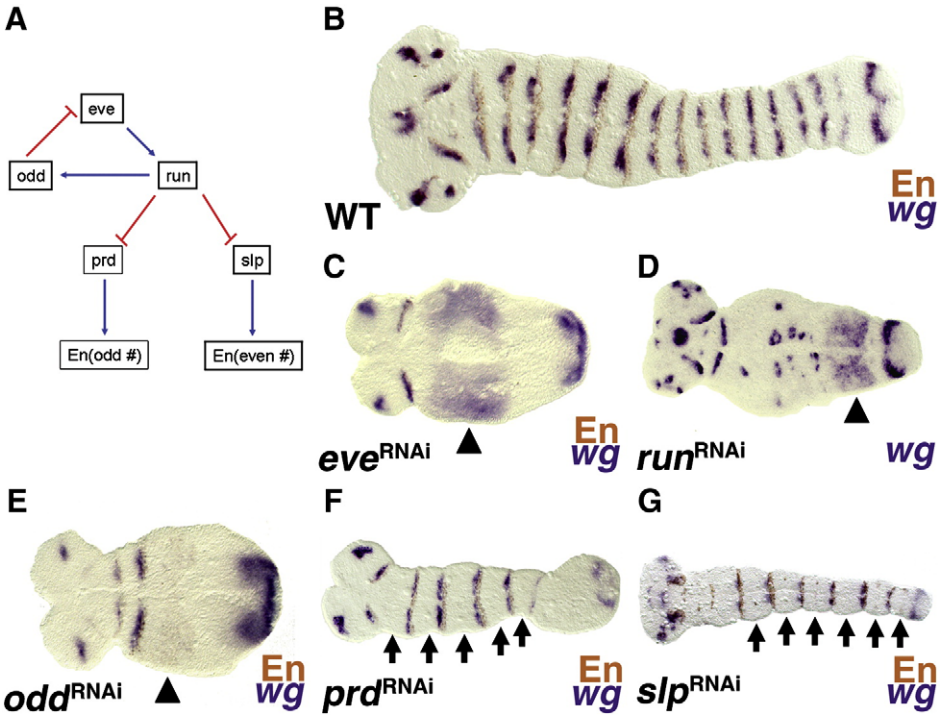


Fig. 1. Expression of Tc-En and *Tc-wg* in *Tribolium* pair-rule gene RNAi embryos. In these ventral views, anterior is to the left. (A) Model of the pair-rule interaction network in *Tribolium*. *Tc-eve*, *Tc-run* and *Tc-odd* comprise a pair-rule gene circuit regulating one another and their downstream targets *Tc-prd* and *Tc-slp* through *Tc-run*. (B) Segmental expression of Tc-En and *Tc-wg* at each parasegmental boundary in wild-type. (C) In this young *Tc-eve*^{RNAi} embryo, the expression of Tc-En is abolished whereas *Tc-wg* (purple, arrowhead) is expressed in a broad central domain instead of stripes. (D) In this *Tc-run*^{RNAi} embryo, *Tc-wg* (purple, arrowhead) stripes are expressed normally in the antennal and mandibular segments *Tc-wg* is also expressed in a broad central domain, instead of in segmental stripes (arrowhead). (E) In this younger *Tc-odd*^{RNAi} embryo, the mandibular and maxillary Tc-En (punctate, brown spots) and *Tc-wg* (purple) stripes form normally. Tc-En (arrowhead) is expressed weakly in a broad central domain in the absence of *Tc-wg* expression. (F) In this *Tc-prd*^{RNAi} embryo, *Tc-wg* (purple) expression is missing in even-numbered parasegments, and Tc-En (punctate, brown spots) is missing in odd-numbered parasegments (arrows). (G) In this *Tc-slp*^{RNAi} embryo, *Tc-wg* (purple) in odd-numbered parasegments is abolished (arrows), but the expression of Tc-En (punctate, brown spots) in even-numbered parasegments is not completely gone.

higher insects (Choe et al., 2006; Davis et al., 2001). Striped expression of pair-rule genes is even more variable among hemimetabolous insects such as *Oncopeltus faciatus* (Liu and Kaufman, 2005), *Gryllus bimaculatus* (Mito et al., 2007), and *Schistocerca americana* (Davis and Patel, 2002; Patel et al., 1994). All together, these results suggest that although pair-rule patterning may be quite ancient within the arthropod lineage, as a developmental mechanism it is quite evolutionarily flexible.

To understand how divergent functions of upstream factors in the segmentation hierarchy ultimately generate the conserved expression patterns of *en* and *wg* required for segment formation, we analyzed pair-rule genes in the red flour beetle *T. castaneum*, whose short-germ mode of embryogenesis is in many ways more typical of development among the Insecta and Arthropoda. Previously, we described a primary pair-rule gene circuit composed of *eve*, *run*, and *odd-skipped* (*odd*) that sequentially prepatterns two segments at a time (Choe et al., 2006), and two functionally complementary secondary pair-rule genes, *paired* (*prd*) and *sloppy-paired* (*slp*), that are responsible for forming odd- and even-numbered segments, respectively (Choe et al., 2006). Furthermore, *h*, *ftz* and *odd-paired* (*opa*), which are key activators of *en* and *wg* expression at the anterior boundary of even-numbered parasegments in *Drosophila* (Benedyk et al., 1994; DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ish-Horowicz et al., 1989), do not appear to function as segmentation genes in *Tribolium* (Choe et al., 2006; Stuart et al., 1991). Despite these differences in pair-rule gene function, stripes of *Tc-en* and *Tc-wg* are expressed at parasegmental boundaries similar to stripes of *en* and *wg* in *Drosophila* (Brown et al., 1994a; Nagy and Carroll, 1994 and Fig. 1B), indicating that within the pair-rule mode of segmentation, different regulatory interactions between pair-rule genes and their segment polarity gene targets have evolved in *Tribolium* and *Drosophila*.

To understand how *Tribolium* pair-rule genes regulate segment polarity genes at parasegmental boundaries, we used RNAi to manipulate the expression of genes in the *Tribolium* pair-rule network. In this network (Fig. 1A), *Tc-eve* is required to activate *Tc-run*, which is required to activate *Tc-odd*, which is then required for periodic repression of *Tc-eve*, sequentially generating primary stripes of *Tc-eve* (Choe et al., 2006). In addition, the secondary pair-rule genes, *Tc-prd* and *Tc-slp*, which occupy parallel positions in this network, are repressed by *Tc-run* (Choe et al., 2006). Severe knock-down of a single primary pair-rule gene results in the complete loss of expression of some and ectopic expression of other genes in this network (Choe et al., 2006). Thus, we were able to examine the results of expressing one or two pair-rule genes in the absence of the others, by performing double or triple RNAi.

When we analyzed the expression of *Tc-en* and *Tc-wg* in *Tribolium* pair-rule gene RNAi embryos, in which only one or two of the known *Tribolium* pair-rule genes were misexpressed, we found that combinations of pair-rule genes different from those in *Drosophila* were required to regulate these segment polarity genes. Expression of the secondary pair-rule genes *Tc-prd* and/or *Tc-slp* in the absence of primary pair-rule gene expression activated *Tc-wg*. *Tc-eve* alone failed to activate *Tc-wg* or *Tc-en*, but in combination with another primary pair-rule gene *Tc-run* or the secondary pair-rule gene *Tc-prd*, in the absence of the other known pair-rule genes, it activated *Tc-en*. Taking into consideration the expression pattern domains of both primary and secondary pair-rule genes in *Tribolium*, we propose two different models for the genetic regulation of the juxtaposed expression of *Tc-wg* and *Tc-en* at odd- and even-numbered parasegmental boundaries, respectively. We also discuss the possibility that the conserved interactions between *eve* and *prd* at the anterior boundary of odd-numbered parasegments may reflect an ancestral segmentation

mechanism that functioned in every segment prior to the evolution of pair-rule segmentation.

Materials and methods

Parental RNAi

Parental RNAi was performed as described (Bucher et al., 2002). 900 ng/μl (*Tc-eve*), 500 ng/μl (*Tc-run*, *Tc-prd* and *Tc-slp*), or 350 ng/μl (*Tc-odd*) of dsRNA was injected into wild-type (GA) pupae to knock down mRNA(s).

Immunocytochemistry and whole-mount in situ hybridization

Immunocytochemistry was carried out as previously described (Patel et al., 1994) with mAbs 2B8 (anti-Eve) diluted 1/20 or 4D9 (anti-En) diluted 1/5 (Developmental Studies Hybridoma Bank at the University of Iowa). Whole-mount in situ was performed with digoxigenin-labeled RNA probes as previously described (Brown et al., 1997).

Results

Effects of primary pair-rule gene RNAi on Tc-En and Tc-wg expression

Previously, we reported that *Tribolium* primary pair-rule genes prepattern two-segment wide regions through a regulatory gene circuit, while secondary pair-rule genes are critical to the formation of the odd- and even-numbered segments through the regulation of *Tc-en* and *Tc-wg* (Choe and Brown, 2007; Choe et al., 2006 and Fig. 1A). After two-segment wide regions are prepatterned, the primary stripes of all three primary pair-rule genes resolve into narrow secondary stripes at the parasegmental boundaries, in cells that will express *Tc-en* (Brown et al., 1997; Choe et al., 2006; Patel et al., 1994). In *Drosophila*, the secondary stripes of *eve*, but not those of *run* or *odd*, are coincident with En. Therefore, it seems likely that the secondary stripes of *Tribolium* primary pair-rule genes function in the regulation of *Tc-en*, *Tc-wg* or both.

To determine how the primary pair-rule genes might regulate *Tc-en* and *Tc-wg*, we analyzed the expression of Tc-En and *Tc-wg* after RNAi of each primary pair-rule gene and determined how it differed from expression in wild-type embryos (Fig. 1B). During normal development, stripes of primary pair-rule genes, secondary pair-rule genes and segment polarity genes are both temporally and spatially dynamic. In younger embryos just beginning germband extension, primary pair-rule stripes of *Tc-eve*, *Tc-run* and *Tc-odd* form in the posterior region of the embryo (Choe et al., 2006). Immediately anterior to this region, the primary stripes resolve into narrow secondary stripes, and *Tc-prd* and *Tc-slp* are activated here (Choe and Brown, 2007; Choe et al., 2006). Slightly more anterior still, where *Tc-prd* resolves into secondary stripes, *Tc-en* and *Tc-wg* are activated in narrow segmental stripes. In older embryos completing germband extension, primary pair-rule stripes have resolved into secondary patterns and/or faded completely while stripes of secondary pair-rule and segment polarity genes are initiated. Also, since there is a gradient of segmental development along the anterior–posterior axis, we might expect Tc-En and *Tc-wg* expression to be completely missing or somewhat expanded in RNAi embryos, but not include the entire length of the embryo. In other words, the effects of expanding or eliminating the expression domain of upstream regulators would be realized in cells that are developmentally competent to express Tc-En and *Tc-wg*.

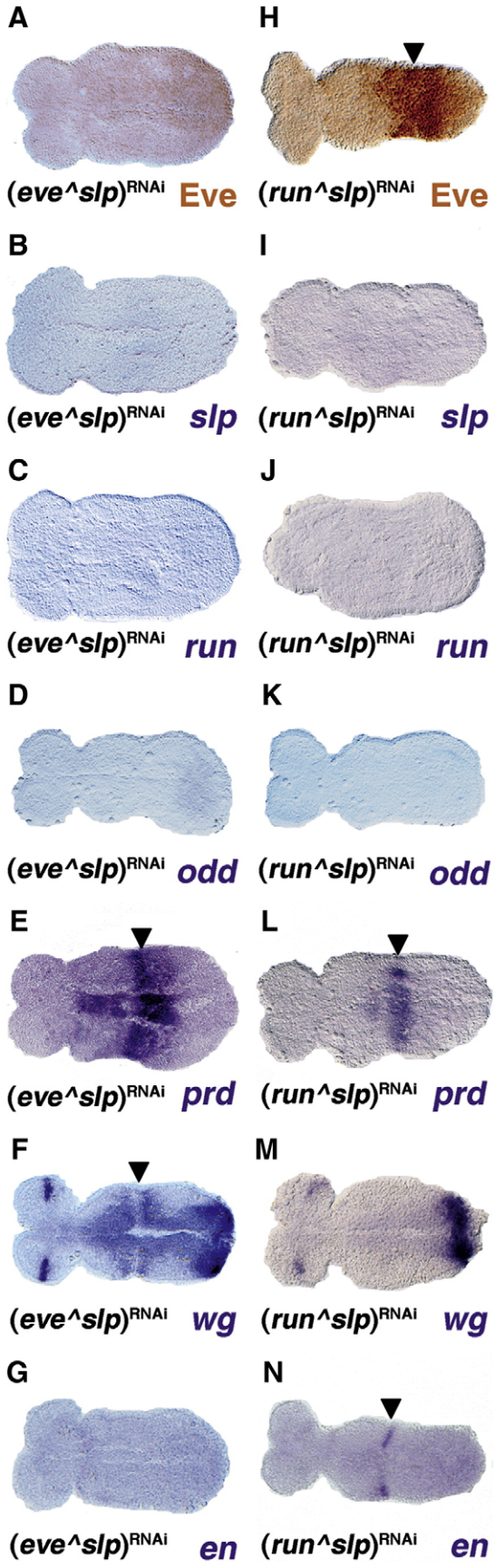
In strong *Tc-eve*^{RNAi} embryos (lacking expression of all three primary pair-rule genes), Tc-En is expressed normally in the preoral head but is not expressed in the segmenting germband, consistent with the development of antennae but not mouth parts or trunk segments in *Tc-eve*^{RNAi} embryonic cuticles (Choe et al., 2006 and Table 1). *Tc-wg* expression, which appeared normal in the preoral head segments and at the posterior end of these embryos, appeared weakly in an expanded domain, rather than in stripes (Fig. 1C, and Table 1). In *Tc-run*^{RNAi} embryos (in which *Tc-eve* is initiated normally and fades from anterior to posterior as the embryos mature, but fails to resolve into primary stripes due to loss of *Tc-run* and *Tc-odd* expression), Tc-En is expressed normally in the preoral head and mandibular segment but is not expressed in the segmenting germband, consistent with development of antennae and mandibles in *Tc-run*^{RNAi} embryonic cuticles (Choe et al., 2006). *Tc-wg* was expressed normally in the preoral head, the mandibular segment and the posterior region of the embryo, but striped expression in the germband was replaced by a broad domain, which appeared more posterior in older embryos (Fig. 1D, and Table 1). Together, these results suggest that *Tc-eve* and *Tc-run* are required for the activation of *Tc-en* and the repression of *Tc-wg*. In strong *Tc-odd*^{RNAi} embryos (in which *both Tc-eve* and *Tc-run* are initiated normally and fade from anterior to posterior as the embryo matures, but fail to resolve into pair-rule stripes), *Tc-en* and *Tc-wg* are expressed normally in the preoral head segments, mandible and maxillary segments, consistent with formation of these segments in embryonic cuticles (Choe et al., 2006 and Fig. 1D). In contrast to the *Tc-eve*^{RNAi} or *Tc-run*^{RNAi} embryos, in *Tc-odd*^{RNAi} striped expression of *Tc-wg* posterior to maxillary

Table 1
Expression of *Tribolium* pair-rule genes, *Tc-en* and *Tc-wg* in RNAi embryos of *Tribolium* pair-rule gene(s)

	eve	*run*	*odd*	*prd*	*slp*	*eve^slp*	*run^slp*	*eve^prd*	*run^prd*	triple
eve	–	+	+	+	+	–	+	–	+	+
run	–	–	+	+	+	–	–	–	–	–
odd	–	–	–	+	+	–	–	–	–	–
prd	+	+	–	–	+	+	+	–	–	–
slp	+	+	–	–	–	–	–	+	+	–
en	–	–	+	–(o)	+	–	+	–	–	–
wg	+	+	–	–(e)	–(o)	+	–	+	–*	–

+ Expression.
– Loss of expression.
* Significantly reduced expression.
(o) Odd-numbered parasegments.
(e) Even-numbered parasegments.
Red gene(s) knocked down by RNAi.
Blue gene examined for expression by in situ hybridization or immunostaining.

segment was completely abolished while Tc-En was expressed weakly in a broad central domain (Fig. 1E, and Table 1), indicating that *Tc-odd* is required for activation of *Tc-wg* and repression of *Tc-en*.



In *Tc-eve*^{RNAi} and *Tc-run*^{RNAi} embryos, *Tc-prd* and *Tc-slp* are expressed in broader than normal domains and fail to resolve into stripes, while in *Tc-odd*^{RNAi} embryos, *Tc-prd* and *Tc-slp* are not activated (Choe et al., 2006 and Table 1). Thus, it is not clear whether misregulation of *Tc-en* and *Tc-wg* in primary pair-rule gene RNAi embryos is a consequence of the loss of direct regulation of segment polarity genes by primary pair-rule genes or an indirect effect through the misregulation of secondary pair-rule genes. To address this question we used RNAi to examine the effects of expressing one or two pair-rule genes in the absence of the other known pair-rule genes.

Combined effects of double RNAi (Tc-eve, Tc-slp) and (Tc-run, Tc-slp) on the expression of Tc-en and Tc-wg

During normal development, the secondary pair-rule gene *Tc-prd* is expressed in cells that will express *Tc-wg* and in cells that will express *Tc-en* (Choe and Brown, 2007). To examine the regulatory activity of *Tc-prd*, we performed double RNAi experiments to knock-down the expression of the other known pair-rule genes. Due to the interactions in the pair-rule gene circuit, performing double RNAi with *Tc-eve* and *Tc-slp* (Figs. 2A–G) produced embryos lacking not only *Tc-eve* (Fig. 2A) and *Tc-slp* (Fig. 2B), but also *Tc-run* (Fig. 2C) and *Tc-odd* (Fig. 2D). In these embryos, *Tc-prd* expression appeared in a broader than normal domain and failed to resolve into stripes (Fig. 2E). *Tc-wg*, but not *Tc-en*, was also expression in an expanded central domain (Figs. 2F, G) suggesting that *Tc-prd* can activate *Tc-wg* but not *Tc-en*, in the absence of the other known pair-rule genes (Table 1).

Using similar pair-rule gene circuit logic, we performed double RNAi with *Tc-run* and *Tc-slp* (Figs. 2H–N). In these embryos, *Tc-eve* failed to resolve into stripes (Fig. 2H), since knocking down *Tc-run* (Fig. 2J) resulted in failure to activate *Tc-odd* (Fig. 2K). *Tc-slp* was knocked down (Fig. 2I) and, as above, *Tc-prd* was expressed in a broader than normal central domain (Fig. 2L). However, *Tc-wg* was not expressed in the central region of these embryos, but *Tc-en* was (compare Figs. 2M and N), suggesting that the combination of *Tc-prd* and *Tc-eve* can activate *Tc-en* independent of the other known pair-rule genes (Table 1).

Combined effects of (Tc-eve, Tc-prd) and (Tc-run, Tc-prd) RNAi on the expression of Tc-wg

In wild-type embryos, *Tc-slp* is expressed in cells that will express *Tc-wg* (Choe and Brown, 2007) suggesting that *Tc-slp* may function in the activation of *Tc-wg*. Furthermore, loss of *Tc-wg* stripes in odd-numbered parasegments in *Tc-slp*^{RNAi} embryos, indicates that *Tc-slp* is a key activator of *Tc-wg* there (Choe and Brown, 2007, and Fig. 1G). To examine the regulatory effects of *Tc-slp* on *Tc-wg* expression, we performed double RNAi with *Tc-eve* and *Tc-prd* (Figs. 3A–F). As a result of knocking down the expression of *Tc-eve* (Fig. 3A), expression of *Tc-run* and *Tc-odd* were also eliminated (Figs. 3B, C). *Tc-prd* was also abolished (Fig. 3D). In the absence of the expression of the other known pair-rule genes, *Tc-slp* was expressed in a broader than normal domain that failed to resolve into stripes (Fig. 3E). In these embryos, *Tc-wg* was expressed more broadly than normal and also failed to

Fig. 2. Analysis of genetic interactions affecting *Tc-en* and *Tc-wg* expression. In these ventral views, anterior is to the left. Double RNAi combinations are denoted by (*x^y*)^{RNAi}. (A–G) (*eve^slp*) double RNAi embryos. In these embryos, the expression of Tc-Eve (expected as punctate brown spots) is abolished (A), as well as the expression of *Tc-run* (purple), *Tc-odd* (purple) and *Tc-slp* (purple) (B-D). *Tc-prd* (purple, arrowhead) expression fails to resolve into stripes (E) and *Tc-wg* (purple, arrowhead) is expressed (F), but *Tc-en* (purple) is not (G). (H–N) (*run^slp*) double RNAi embryos. In the (*run^slp*) double RNAi embryos, Tc-Eve (punctate, brown spots, arrowhead) fails to resolve into stripes (H), while *Tc-run* (purple), *Tc-odd* (purple) and *Tc-slp* (purple) are not expressed (I–K). *Tc-prd* (purple, arrowhead) is expressed in a broader than normal domain and *Tc-wg* (purple) expression in the trunk is not initiated (M) but *Tc-en* (purple, arrowhead) is (N). In (G, N), the young embryos shown have not yet developed *Tc-en* expression in the antennae.

form stripes (Fig. 3F). Thus, independent of the other known pair-rule genes, *Tc-slp* activated *Tc-wg* (Table 1).

To examine the regulatory ability of *Tc-slp* in the presence of *Tc-eve* but not the other known pair-rule genes, we performed double RNAi with *Tc-run* and *Tc-prd* (Figs. 3G–L). In these embryos, *Tc-eve* failed to resolve into pair-rule stripes (Fig. 3G), since knocking down *Tc-run* expression (Fig. 3H), eliminated the expression of *Tc-odd* (Fig. 3I). *Tc-prd* was knocked down (Fig. 3J) and, as above, *Tc-slp* was expressed over a broader than normal primary domain and failed to resolve into stripes (Fig. 3K). Expression of *Tc-wg* was restricted to the anterior-most region of the ectopic *Tc-slp* domain and was fainter than in *Tc-eve*, *Tc-prd* double RNAi (compare Figs. 3F and L). Thus, independent of the other known pair-rule genes, expression of *Tc-eve* in addition to *Tc-slp* had a repressive effect on the expression of *Tc-wg* (Table 1).

Regulatory actions of Tc-eve and Tc-run in the absence of secondary pair-rule genes

In the even-numbered parasegments of wild-type embryos the primary pair-rule genes *Tc-eve*, *Tc-run* and *Tc-odd*, as well as the secondary pair-rule gene *Tc-prd* are continuously expressed in cells that will express *Tc-en* (Brown et al., 1997; Choe and Brown, 2007; Choe et al., 2006; Patel et al., 1994). The temporal and spatial relationships between the expression of these genes and *Tc-en*, suggest that one, some, or all of them are required to activate *Tc-en* here. *Tc-prd*^{RNAi} revealed that *Tc-prd* is not required for Tc-En expression in these stripes (Choe et al., 2006 and Fig. 1F). Furthermore, *Tc-odd* is required to repress *Tc-en* rather than to activate it (Fig. 1E). In *Tc-odd*^{RNAi} embryos, *Tc-eve* and *Tc-run* are ectopically expressed in the absence of *Tc-prd* and *Tc-slp* (Choe et al., 2006) suggesting that either *Tc-eve*, *Tc-run* or both are required to activate *Tc-en* (Table 1).

To determine if *Tc-eve* expression can activate *Tc-en* independent of the other pair-rule genes, we performed *Tc-run*, *Tc-prd*, *Tc-slp* triple RNAi. Since *Tc-eve*, *Tc-prd* and *Tc-slp* are expressed in *Tc-run*^{RNAi} embryos (Choe et al., 2006), we expected that only *Tc-eve* would be expressed in the triple RNAi embryos. Indeed, *Tc-eve* was expressed broadly and did not resolve into stripes in the absence of the other pair-rule genes in the triple RNAi embryos (Figs. 4A–E). If *Tc-eve* was sufficient to activate *Tc-en*, we expected *Tc-en* to be expressed in the triple RNAi embryos, but it was not (Fig. 4F), indicating that although *Tc-eve* is required, it was not sufficient to activate *Tc-en* (Table 1).

Unfortunately, with our current approaches to manipulate the expression of pair-rule genes via RNAi, we could not express *Tc-run* in the absence of the other genes to test whether *Tc-run* is sufficient to activate *Tc-en*. The overexpression of *Tc-run* might show whether *Tc-run* is sufficient to activate *Tc-en*. However, two pieces of evidence, the loss of Tc-En expression in *Tc-run*^{RNAi} embryos and the ectopic expression of Tc-En when *Tc-eve* and *Tc-run* are ectopically expressed in the absence of the secondary pair-rule genes, strongly suggest that *Tc-run* is required to activate *Tc-en* and, while it may require input from *Tc-eve* or other genes, this activation does not require additional input from *Tc-prd* and *Tc-slp*.

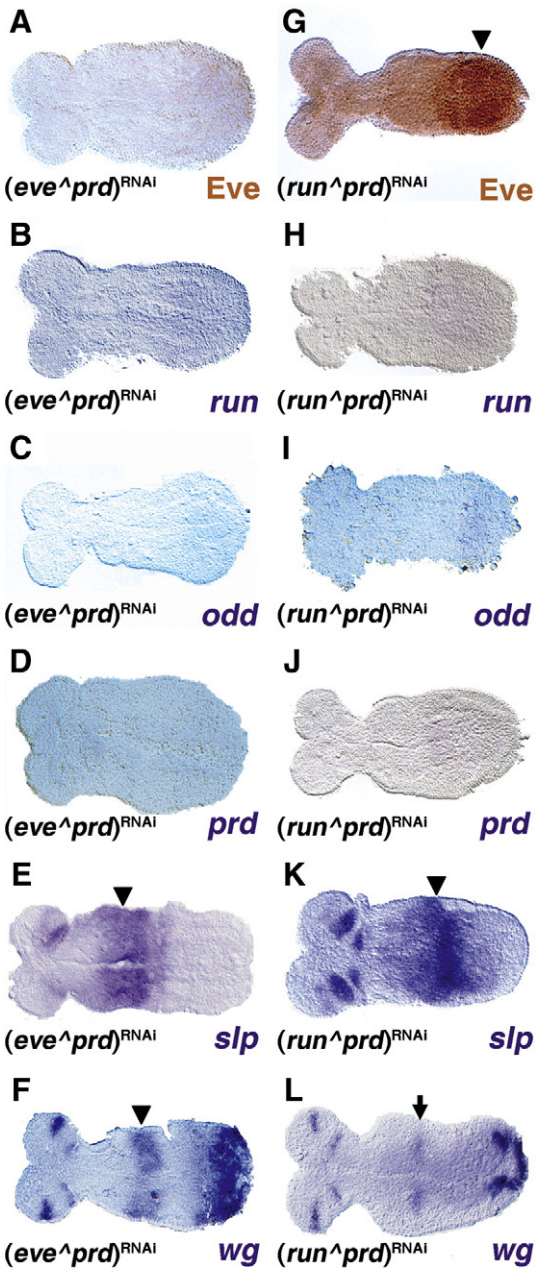


Fig. 3. Analysis of genetic interactions affecting *Tc-wg* expression. (A–F) (*eve^prd*) double RNAi embryos. In these embryos, Tc-Eve (expected in punctate, brown spots) is not expressed (A) which results in the loss of *Tc-run* (purple, B), and *Tc-odd* (purple, C). *Tc-prd* is abolished (purple, D). *Tc-slp* (purple, arrowhead) fails to resolve into stripes (E) and *Tc-wg* (purple, arrowhead) is expressed (F). (G–L) (*run^prd*) RNAi embryos. In these embryos, Tc-Eve (punctate, brown spots, arrowhead) fails to resolve into stripes (G) since the expression of *Tc-run* (purple) and thus *Tc-odd* (purple) are abolished (H, I). *Tc-prd* (purple) expression is also abolished (J) and *Tc-slp* (purple, arrowhead) is expressed broadly rather than in stripes (K). However, *Tc-wg* (purple, arrow) is expressed weakly in a very narrow region in the anterior region of the *Tc-slp* domain (L).

Discussion

Using RNAi to manipulate the expression of the five genes known to provide pair-rule function in *Tribolium*, such that only one or two of them are expressed in the absence of the others, provides new insights into the genetic mechanisms by which *Tribolium* pair-rule genes regulate the expression of the segment polarity genes *Tc-en* and *Tc-wg*. The secondary pair-rule genes *Tc-prd* and *Tc-slp* activated *Tc-wg* independent of the primary pair-rule genes, but when they were expressed in combination with *Tc-eve*, *Tc-wg* was repressed (Figs. 2 and 3). *Tc-eve*, independent of the other pair-rule genes, did not activate the segment polarity genes, but in combination with *Tc-run* or *Tc-prd* it activated *Tc-en*. By considering these results in the context of the expression patterns of these genes during normal development it is possible to generate a model for the genetic regulation of *Tc-en* and *Tc-wg* in *Tribolium* in which different pair-rule gene interactions define the anterior boundaries of odd- and even-numbered parasegments, respectively.

Genetic regulation of Tc-en and Tc-wg by Tribolium pair-rule genes

During normal development the primary pair-rule genes *Tc-eve*, *Tc-run* and *Tc-odd* are each expressed in eight primary pair-rule

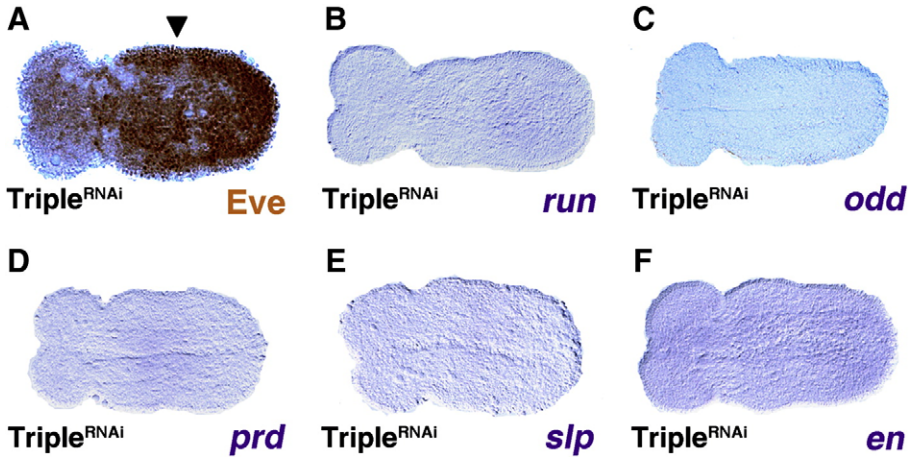


Fig. 4. Expression of *Tc-en* and pair-rule genes in *Tc-run*, *Tc-prd*, *Tc-slp* triple RNAi embryos. In these embryos, Tc-Eve (punctate, brown spots, arrowhead) fails to resolve into stripes (A) since *Tc-run* (purple), and thus *Tc-odd* (purple) expression was successfully knocked down (B, C). *Tc-prd* (purple) and *Tc-slp* (purple) expression was also successfully abolished (D, E). *Tc-en* (purple) is not expressed (F).

stripes. *Tc-eve* primary stripes resolve into 16 secondary stripes, each of which is located at the anterior boundary of a parasegment (Brown et al., 1994a; Choe et al., 2006; Patel et al., 1992). These secondary *Tc-eve* stripes are designated *eve* a and *eve* b in Fig. 5A. *Tc-run* resolves into eight secondary stripes (2° in Fig. 5A), located at the anterior boundary of even-numbered parasegments (Choe et al., 2006). The secondary pair-rule gene, *Tc-prd*, is initially expressed in seven stripes, some of which resolve into secondary stripes. Seven of these are co-expressed with the secondary stripes of *Tc-eve* and *Tc-run* at the anterior boundary of the even-numbered parasegments (Choe and Brown, 2007) (*prd* b in Fig. 5A). The other *Tc-prd* secondary stripes are slightly wider, and span the boundary at the anterior border of odd-numbered parasegments (*prd* a in Fig 5A). Two stripes of *Tc-slp* expression appear simultaneously, each positioned at the posterior border of a segment (Choe and Brown, 2007) (labeled *slp* a and *slp* b in odd and even-numbered parasegments, respectively, in Fig. 5A).

Based on these results, we suggest that two different genetic mechanisms regulate *Tc-en* and *Tc-wg* at the anterior boundary of odd- and even-numbered parasegments, respectively. To define the anterior boundary of odd-numbered parasegments, *Tc-en* is activated in cells expressing both *Tc-eve* and *Tc-prd* (denoted by blue activation lines in Fig. 5A). This broader secondary stripe of *Tc-prd* also activates *Tc-wg*, but this activation is repressed by *Tc-eve* (denote by red repression lines in Fig. 5A), restricting expression of *Tc-wg* to cells in even-numbered parasegments immediately anterior to stripes of *Tc-en* expression. Therefore, *Tc-eve* appears to be required as a repressor of *Tc-wg* and, in addition to the secondary pair-rule gene, *Tc-prd*, as a coactivator of *Tc-en* (*Tc-prd* did not activate *Tc-en* in the absence of the other known pair-rule genes, Fig. 2G) to generate the juxtaposed stripes of *Tc-en* and *Tc-wg* that ultimately define the boundary between *Tc-en* in an odd-numbered parasegment and *Tc-wg* in the anterior even-numbered parasegment.

To define the other parasegmental boundary, between *Tc-en* in even-numbered parasegments and *Tc-wg* in odd-numbered parasegments, *Tc-en* is activated by the overlapping secondary stripes of *Tc-run* and *Tc-eve* (*Tc-eve* b). *Tc-slp* (*Tc-slp* a) activates *Tc-wg*, but this activation appears to be repressed by *Tc-eve*, restricting *Tc-wg* expression to cells in odd-numbered parasegments immediately anterior to the *Tc-en* stripes. Thus, *Tc-eve* and *Tc-run*, act as coactivators of *Tc-en*, and *Tc-eve* acts to repress *Tc-wg* expression to define the anterior boundary of even-numbered parasegments.

Previously, we described the *Tribolium* primary pair-rule gene circuit, composed of *Tc-eve*, *Tc-run* and *Tc-odd*, which generates eight stripes to prepattern regions of double segment periodicity in the posterior region of the growth zone, and regulates *Tc-prd* and *Tc-slp*, which are important to form odd- and even-numbered segments, respectively (Choe et al., 2006). In our current model of the regulation of *Tc-en* and *Tc-wg* by *Tribolium* pair-rule genes, we suggest that the continuous expression of the primary pair-rule genes, *Tc-eve* and *Tc-run*, is required in addition to *Tc-prd* and *Tc-slp* to regulate *Tc-en* and *Tc-wg*. In contrast, in *Drosophila*, the primary pair-rule gene *eve* acts indirectly through secondary pair-rule genes to regulate segment polarity genes (Coulter and Wieschaus, 1988; DiNardo and O'Farrell, 1987; Fujioka et al., 1995; Fujioka et al., 2002; Jaynes and Fujioka, 2004; Manoukian and Krause, 1992), while the secondary stripes of the primary pair-rule gene *run* act directly to repress *en* (Aronson et al., 1997; Manoukian and Krause, 1993). In *Tribolium*, *Tc-eve* and *Tc-run* may control additional, as yet unidentified, secondary pair-rule genes to regulate *Tc-en* and *Tc-wg*. Alternatively, we are intrigued by the possibility that *Tc-eve* and *Tc-run* may function directly with the secondary pair-rule genes *Tc-prd* and *Tc-slp* to regulate the segment polarity genes, which may account for a seemingly smaller complement of pair-rule genes in *Tribolium* relative to *Drosophila*.

Comparison of the genetic mechanisms defining parasegmental boundaries in Drosophila and Tribolium

In both insects, the regulation of *en* and *wg* expression at the anterior boundary of odd-numbered parasegments differs from the regulation of *en* and *wg* at the anterior boundary of even-numbered parasegments (Fig. 5). In principle, the genetic mechanism defining the anterior boundary of odd-numbered parasegments is conserved between *Drosophila* and *Tribolium*. For example, in *Drosophila*, as in *Tribolium*, *en* is activated by *prd* within the *eve* expression domain in odd-numbered parasegments (Ingham et al., 1988; Morrissey et al., 1991). In addition, in both insects, *prd* is sufficient to activate the adjacent *wg* stripe in even-numbered parasegments (Fig. 2 and Cadigan et al., 1994; DiNardo and O'Farrell, 1987; Fujioka et al., 1996).

Although *eve* is required for the activation of *en* in *Tribolium*, we do not know whether it acts directly on *en* or through other pair-rule genes. In *Drosophila*, *eve* plays a permissive role in the activation of *en*, by regulating the expression of *prd* and *slp* (Fujioka et al., 1995). That is, *eve* represses *slp*, positioning the posterior boundary of *slp* at the posterior border of even-numbered parasegments (Fujioka et al., 1995; Jaynes and Fujioka, 2004). Furthermore, high concentrations of *eve* in odd-numbered parasegments repress *prd* to define the posterior border of *prd* expression (Baumgartner and Noll, 1990; Fujioka et al., 1995). Thus, by defining the posterior boundaries of *slp* and *prd*

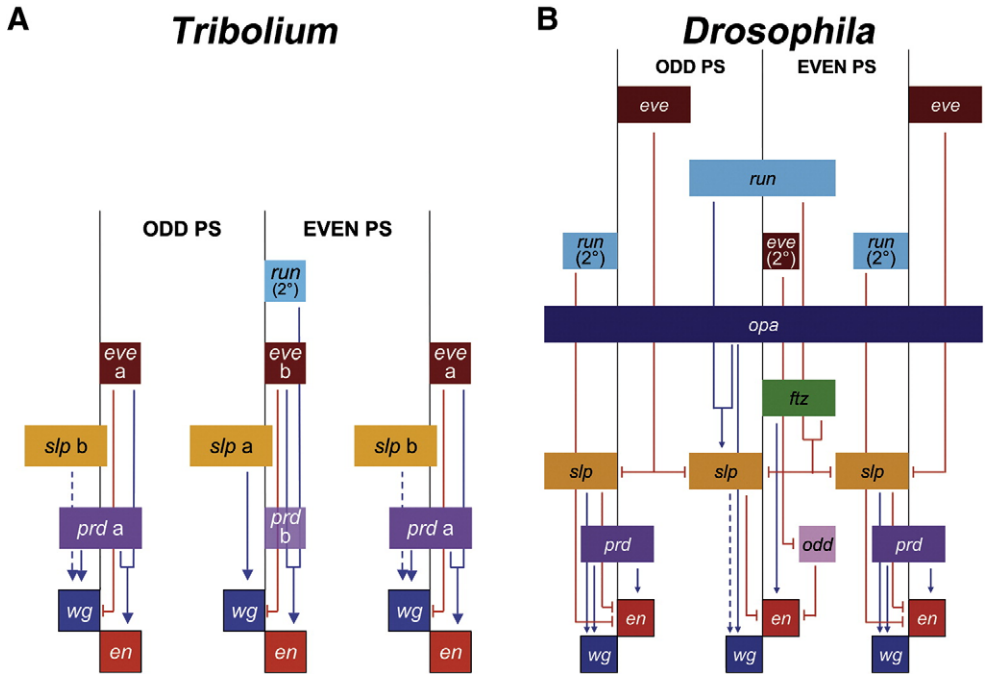


Fig. 5. Modeling the regulation of *en* and *wg* by pair-rule genes in *Tribolium* and *Drosophila*. (A) Regulation of *Tc-en* and *Tc-wg* by *Tribolium* pair-rule genes. The secondary stripes of *Tc-eve* and *Tc-prd* are required to activate *Tc-en* in odd-numbered parasegments. *Tc-prd* is required to activate the adjacent stripe of *Tc-wg* in even-numbered parasegments. The secondary stripes of *Tc-eve* and *Tc-run* are required to activate *Tc-en* in even-numbered parasegments. *Tc-slp* a is required to activate *Tc-wg* in odd-numbered parasegments. *Tc-eve* also represses the expression of *Tc-wg* in the anterior region of every parasegment where *Tc-en* is expressed. (B) Summary of the basic regulation of *en* and *wg* by *Drosophila* pair-rule genes. *en* in odd-numbered parasegments is activated by *prd* while *wg* in even-numbered parasegments is activated by *prd* and *slp*. *eve* in odd-numbered parasegments represses the expression of *slp*. *slp* also represses *en* in the even-numbered parasegments. Secondary *run* stripes repress *en* in the even-numbered parasegments. *en* in even-numbered parasegments is activated by *ftz*, while *wg* in odd-numbered parasegments is activated by *opa*. *eve* in even-numbered parasegments represses *odd*. *odd* represses *en* in even-numbered parasegments. *run* in combination with *opa* activates *slp* in odd-numbered parasegments whereas *run* in combination with *ftz* represses *slp* in even-numbered parasegments. *slp* also represses *en* in odd-numbered parasegments and maintains *wg* in even-numbered parasegments. Activation and repression are in blue and red lines, respectively, and maintenance interactions are denoted by broken blue lines.

expression, *eve* determines the *en* expression domain. In this scenario, *eve* functions to permit *prd* activation of *en* rather than as a coactivator of *en*.

The expression domains of *slp* and *run* and their functions in the regulation of *en* and *wg* do not appear to be conserved between *Drosophila* and *Tribolium*. The register of primary *slp* stripes in *Tribolium* (Choe and Brown, 2007) is opposite that in *Drosophila* (Grossniklaus et al., 1992) and, in *Drosophila*, but not in *Tribolium*, *slp* is required, in addition to *prd*, to activate *wg* in the even-numbered parasegments (Cadigan et al., 1994). In addition, *slp* also represses *en* in these cells in *Drosophila* (Cadigan et al., 1994; Jaynes and Fujioka, 2004). However, in *Tribolium*, *Tc-slp* does not repress *Tc-en*; *Tc-en* is initiated normally in severe *Tc-slp*^{RNAi} embryos (Choe and Brown, 2007). In *Drosophila*, secondary *run* stripes are coincident with *wg* stripes, and function to repress *en* in these cells (Aronson et al., 1997; Manoukian and Krause, 1993). In contrast, the eight primary stripes of *Tc-run* resolve into eight secondary stripes that are coincident with *Tc-en* in even-numbered parasegments. They do not overlap with *Tc-wg* in even-numbered parasegments (Brown and Denell, 1996) and thus cannot repress *Tc-en* there.

To define the other parasegmental boundary, between *en* in even-numbered parasegments and *wg* in odd-numbered parasegments, *Drosophila* and *Tribolium* use different regulatory mechanisms. In *Drosophila*, *ftz* and *opa* are key activators of *en* in even-numbered parasegments and *wg* in odd-numbered parasegments, respectively (Benedyk et al., 1994; DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ish-Horowicz et al., 1989). Furthermore, the minor secondary *eve* stripes are important to *ftz*-dependent *en* activation in even-numbered parasegments by repressing *odd*, which represses *en* (Coulter and Wieschaus, 1988; DiNardo and O'Farrell, 1987; Fujioka et al., 1995; Manoukian and Krause, 1992). In addition to *eve*, *run* also indirectly regulates *en* at the parasegmental boundary; *run* in combination with *opa*, activates *slp* at the posterior border of odd-numbered parasegments to repress *en* there, whereas *run* in combination with *ftz* represses *slp* in even-numbered parasegments to permit *ftz*-dependent *en* activation (Fig. 5B and Swantek and Gergen, 2004), Thus, in *Drosophila*, primary pair-rule genes *eve* and *run* indirectly regulate *en* in even-numbered parasegments by regulating secondary pair-rule genes (Fig. 5B). However, *Tc-ftz* and *Tc-opa* are not functional in *Tribolium* segmentation (Choe et al., 2006; Stuart et al., 1991). *Tc-slp*, instead of *Tc-opa*, activates *Tc-wg* in odd-numbered parasegments (Choe and Brown, 2007) while the primary pair-rule genes, *Tc-eve* and *Tc-run*, instead of *Tc-eve* and *Tc-ftz*, activate *Tc-en* in even-numbered parasegments. Furthermore, *Tc-slp* activation of *Tc-wg* is suppressed by *Tc-eve* (Fig. 3) and *Tc-slp* expression is repressed by *Tc-run* (Choe et al., 2006). Therefore, in *Tribolium*, primary pair-rule genes *Tc-eve* and *Tc-run*, with the secondary pair-rule gene *Tc-slp*, are required for proper regulation of *Tc-en* in even-numbered parasegments and adjacent *Tc-wg* in odd-numbered parasegments (Fig. 5A). Interestingly, *en* is expressed in cells expressing secondary stripes of *prd* in even-numbered parasegments of both *Drosophila* and *Tribolium*. However, *prd* does not seem to be involved in the regulation of these *en* stripes in either insect since they are not disrupted in a *Drosophila prd* null mutant or in severe *Tribolium prd*^{RNAi} embryos (Choe and Brown, 2007; DiNardo and O'Farrell, 1987).

Conserved and divergent pair-rule gene functions in the regulation of en and wg

In both *Drosophila* and *Tribolium*, *wg* expression is positively regulated by *prd* and *slp*. Using RNAi in *Tribolium* to knockdown the expression of all known pair-rule genes except *Tc-prd* (Fig. 2) or *Tc*-slp (Fig. 3) results in the expression of *Tc-wg*. In *Drosophila*, the continued

expression of *wg* in *slp* mutants suggests *prd* is an activator of *wg*, while the overexpression of *slp* induces ectopic expression of *wg*, indicating *slp* is also an activator of *wg* (Cadigan et al., 1994).

In both *Drosophila* and *Tribolium*, *eve* is required in combination with *prd* to activate *en*. In *Tribolium*, using RNAi to knockdown all known pair-rule genes except *eve* and *prd* results in the expression of *Tc-en* (Fig. 2). In *Drosophila*, ectopic expression of *prd* induces expression of *en* only in the *eve* expression domain (Morrissey et al., 1991).

Regulating the juxtaposed stripes of *en* and *wg* is critical to define the parasegmental boundaries in insects and probably all arthropods. As we have shown, the genetic mechanism defining the anterior boundary of odd-numbered parasegments is similar in *Drosophila* and *Tribolium*. *prd* is required to activate *en* and *wg* in both insects. In *Drosophila*, *slp* is also required to activate *wg* and repress *en*. Finally, *eve* represses *slp* to produce the adjacent stripes of *en* and *wg*. In *Tribolium*, *Tc-eve* suppresses *Tc-prd* activation of *Tc-wg*, to produce adjacent stripes of *Tc-en* and *Tc-wg*. Thus, in the regulation of *en* and *wg*, repression of *wg* appears to be critical in *Tribolium*, while repression of *en* is critical in *Drosophila* (Fig. 5).

Previously, we determined that not all orthologs of *Drosophila* pair-rule genes participate in *Tribolium* segmentation (Choe et al., 2006). The limits of this candidate gene approach raised the possibility that the function of additional pair-rule genes is necessary to explain the conserved segmental expression of *en* and *wg* at parasegmental boundaries (Choe et al., 2006). Indeed, this possibility still cannot be ruled out. Alternative approaches to identify novel pair-rule genes in *Tribolium* and continued comparative analysis of segmentation in other insects is required to determine whether the pair-rule gene regulation of segment polarity genes described here represents a general mode of segmentation or is specific to *Tribolium*.

Insights into pair-rule gene regulation of en and wg in insect evolution

Pair-rule gene expression is highly variable among nondrosophilid insects and basally branching arthropods suggesting that the regulatory input to the segment polarity genes must be significantly modified in different lineages (Peel et al., 2005). Computational modeling of the segment polarity gene network indicates that it is a developmental module that is likely to be resistant to variations in regulatory inputs (von Dassow et al., 2000), but does not explain of how such variations might function or evolve. Our studies provide functional evidence that the *Tribolium* pair-rule gene network and the regulatory input it provides to segment polarity genes differ from *Drosophila*, yet still produce the highly conserved pattern of *en* and *wg* expression to define parasegmental boundaries.

Repression of primary *eve* stripes into secondary stripes differs between *Drosophila* and *Tribolium*. In *Drosophila*, primary stripes fade from the posterior and expression of *eve* is renewed in even-numbered parasegments (Macdonald et al., 1986). In *Tribolium*, *Tc-eve* primary stripes split into secondary stripes by repression in the middle of the primary stripes by an as yet unknown mechanism; *Tc-eve* is continuously expressed in every parasegment (Brown et al., 1997; Patel et al., 1994). This difference in expression dynamics led us to hypothesize that *Tc-eve* may play a similar role in every parasegment in *Tribolium*, even though it performs different functions in odd- and even-numbered parasegments in *Drosophila*. In our current model, unlike in *Drosophila*, the requirements for *Tc-eve* activity are the same in every segment in that it is required for repression of *Tc-wg* and, in combination with a coactivator (*Tc-prd* or *Tc-run*), for activation of *Tc-en*.

Interestingly, *eve* expression is highly variable among insects. It is expressed only in pair-rule stripes in some insects, in both pair-rule and segmental stripes in others, and only in segmental strips in still other insects (Liu and Kaufman, 2005). However, *eve* is expressed in segmental, not pair-rule, stripes in other arthropods (Damen et al., 2005; Liu and Kaufman, 2005). Thus it is likely that its ancestral pattern was segmental in insects. In contrast, *prd* expression in pair-rule stripes is largely conserved in insects (Choe and Brown, 2007; Davis et al., 2005; Davis et al., 2001; Kilchherr et al., 1986; Osborne and Dearden, 2005). In *Drosophila* and *Tribolium*, *prd* is required to activate *en* and *wg*, while *eve* is required to activate *en* and repress *wg* at the anterior boundary of odd-numbered parasegments. These regulatory interactions might represent an ancestral mechanism that functioned in every parasegment, but is retained only in odd-numbered parasegments in these two insects. We provide a simple model describing how these genes might have regulated segment polarity genes in an ancestral pair-rule mechanism, which relies on segmental stripes of *eve* and pair-rule stripes of *prd* (Fig. 6A). In this model, *prd* activates *en* and *wg*, while *eve* is required to activate *en* and repress *wg*. The segmental stripes of *eve*, which are expressed first, are poised to repress *prd* activation of *wg* in the *en* expressing cells on the posterior side of each parasegmental boundary. Further, we can adapt this model to explain how the segmental stripes of both *prd* and *eve* might regulate the expression of *wg* and *en* via an ancestral segmentation mechanism (Fig. 6B). In this model, the segmental *prd* stripes extend more anterior than those of *eve*. Again previous expression of *eve* would be poised to repress *prd* activation of *wg*. This

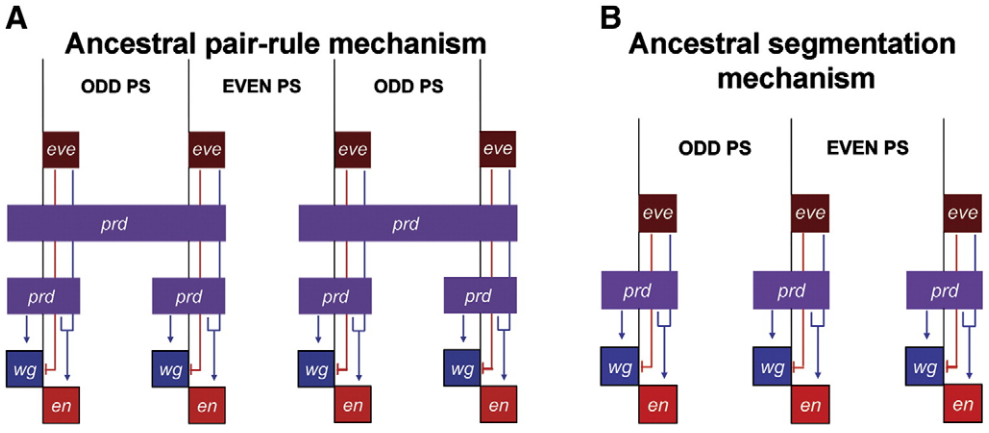


Fig. 6. Modeling pair-rule regulation of *en* and *wg* in ancestral insects and arthropods. (A) Putative regulation of *en* and *wg* by segmental stripes of *eve* and pair-rule stripes of *prd* in ancestral insects. In this model, pair-rule stripes of *prd* would prepattern units that are two-segment wide that are then resolved into segmental stripes. These segmental stripes activate *en* and *wg* at each parasegmental boundary while segmental stripes of *eve*, coincident with *en* stripes, would suppress *prd*-dependent *wg* activation. (B) Putative regulation of *en* and *wg* by segmental stripes of *eve* and *prd* in basally branching arthropods. In this model, segmental stripes of *prd* would overlap both *en* and *wg* stripes while segmental stipes of *eve* are coincident with *en* stripes. *prd* would activate *en* and *wg* whereas *eve* would suppress the activation of *wg* by *prd* in *en* expressing cells.

proposed segmentation mechanism is consistent with the repeating striped expression of *eve* or *prd* orthologs in other arthropods (Peel et al., 2005). While the specific genes involved may vary between lineages, (for example, the ortholog of *eve* is not expressed in segmental stripes in *Schistocerca* (Patel et al., 1992)), the basic principle of two overlapping segmental expression domains, one capable of activating *wg* and *en*, and the other more posterior domain repressing *wg*, may constitute a generic arthropod segmentation mechanism.

It is important to note that we have considered pair-rule inputs to segment polarity genes, and not requirements to activate or regulate the pair-rule genes themselves. While the ancestral segmentation model does not employ a pair-rule mechanism per se, it describes a system that might have evolved into the pair-rule systems found in *Drosophila* and *Tribolium*, and perhaps other insects. Comparative analysis of pair-rule regulation of the segment polarity genes in basal insects and arthropods will provide the necessary tests of these models.

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