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Evolutionary flexibility of pair-rule patterning revealed by functional analysis of secondary pair-rule genes, *paired* and *sloppy-paired* in the short-germ insect, *Tribolium castaneum*

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Abstract

In the *Drosophila* segmentation hierarchy, periodic expression of pair-rule genes translates gradients of regional information from maternal and gap genes into the segmental expression of segment polarity genes. In *Tribolium*, homologs of almost all the eight canonical *Drosophila* pair-rule genes are expressed in pair-rule domains, but only five have pair-rule functions. *even-skipped*, *runt* and *odd-skipped* act as primary pair-rule genes, while the functions of *paired* (*prd*) and *sloppy-paired* (*slp*) are secondary. Since secondary pair-rule genes directly regulate segment polarity genes in *Drosophila*, we analyzed *Tc-prd* and *Tc-slp* to determine the extent to which this paradigm is conserved in *Tribolium*. We found that the role of *prd* is conserved between *Drosophila* and *Tribolium*; it is required in both insects to activate *engrailed* in odd-numbered parasegments and *wingless* (*wg*) in even-numbered parasegments. Similarly, *slp* is required to activate *wg* in alternate parasegments and to maintain the remaining *wg* stripes in both insects. However, the parasegmental register for *Tc-slp* is opposite that of *Drosophila slp1*. Thus, while *prd* is functionally conserved, the fact that the register of *slp* function has evolved differently in the lineages leading to *Drosophila* and *Tribolium* reveals an unprecedented flexibility in pair-rule patterning.

Keywords: *paired*; *sloppy-paired*; Segmentation; Pair-rule gene; *Tribolium castaneum*

Introduction

Genetic studies of the segmented body plan in *Drosophila* and vertebrates have detailed two different segmentation mechanisms; the spatial regulation of segmentation genes by a genetic hierarchy that produces segments simultaneously in *Drosophila* (Ingham, 1988) and the temporal regulation of segmentation components by a segmentation clock that produces somites sequentially in vertebrates (Pourquie, 2003). While long-germ embryogenesis in *Drosophila* is considered to be a derived mode, most other insects display short-germ embryogenesis in which most segments are added sequentially. Because of the morphological similarity of sequential segmentation to vertebrate somitogenesis, temporal as well as spatial regulation of the segmentation process in short-germ insects and other basal arthropods has been the focus of many recent studies. Although evidence for a segmentation clock has been described for basal arthropods (Chipman et al., 2004; Stollewerk et al., 2003), there is as yet no such evidence for insects. In contrast, comparative studies on homologs of *Drosophila* segmentation genes in other insects have revealed that a fairly conserved hierarchical cascade of genes spatially regulates segmentation. For example, segmental expression patterns of segment polarity genes are conserved in all arthropods examined thus far (Damen et al., 1998; Nulsen and Nagy, 1999). However, despite the importance of pair-rule genes as translators of nonperiodic information from maternal and gap genes to the periodic expression of segment polarity genes in *Drosophila* (Niessing et al., 1997), homologs of the pair-rule genes show the most diverse expression patterns, from typical pair-rule expression to expression in every segment or even nonsegmental expression in other short-germ insects (Davis and Patel, 2002; Dawes et al., 1994; Liu and Kaufman,

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2005; Patel et al., 1992). Furthermore, the systematic RNAi analysis of *Tribolium* homologs of *Drosophila* pair-rule genes that are expressed in a pair-rule manner, revealed various segmental phenotypes, from asegmental to typical pair-rule (Choe et al., 2006). Others failed to affect segmentation, confirming previous observations that expression patterns are not always consistent with function (Brown et al., 1994; Stuart et al., 1991). We observed typical pair-rule phenotypes when analyzing the homologs of two *Drosophila* secondary pair-rule genes (*paired* and *sloppy-paired*), leading us to hypothesize that these might be the best candidate genes to test the extent to which pair-rule mechanisms are conserved in arthropod segmentation.

In *Drosophila* blastoderm stage embryos, pair-rule genes initiate and maintain expression of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) at the parasegmental boundaries to molecularly define segments (Jaynes and Fujioka, 2004; Nasiadka et al., 2002). Immediately after gastrulation, the expression of *en* and *wg* are mutually dependent upon one another to maintain parasegmental boundaries and to ultimately form segmental grooves (Martinez Arias et al., 1988).

Drosophila paired (*prd*), one of the earliest pair-rule genes identified, has been analyzed in detail (Frigerio et al., 1986; Kilchherr et al., 1986; Morrissey et al., 1991). It functions at the end of the pair-rule gene network as a direct activator of the segment polarity genes *en* and *wg* (Baumgartner and Noll, 1990), and a null allele produces an obvious pair-rule phenotype in which all odd-numbered trunk segments are missing (Coulter and Wieschaus, 1988). Due to these features of *prd*, homologs of *Drosophila prd* or Pax group III genes have been analyzed in various insects and some basal arthropods to understand pair-rule patterning (Davis et al., 2001; Dearden et al., 2002; Osborne and Dearden, 2005; Schoppmeier and Damen, 2005). Indeed, all known homologs of *prd* or Pax group III genes displayed pair-rule expression patterns in insects suggesting that *prd* is an ancient pair-rule gene. However, this hypothesis has yet to be functionally tested.

Drosophila has two *sloppy-paired* (*slp*) genes, *slp1* and *2*, which display almost identical expression patterns and are functionally redundant (Cadigan et al., 1994a; Grossniklaus et al., 1992). In contrast to the clear pair-rule phenotype of *prd* null mutants, embryos lacking both *slp1* and *2* display various segmental phenotypes ranging from pair-rule to the lawn of denticles produced by *wg*-class segment polarity genes as well as gap-like phenotypes in the head (Grossniklaus et al., 1994; Grossniklaus et al., 1992). *slp1* and *2* are required to activate *wg* and repress *en*. Similar to *prd*, *slp* mutants that display pair-rule phenotypes are defective primarily in odd-numbered segments (Grossniklaus et al., 1992). Because of these phenotypic variations and its functional similarity to *prd*, homologs of *Drosophila slp* have not been the focus of evolutionary studies for understanding pair-rule patterning in other insects and arthropods. Only one study, on the segmental expression of the *slp* homolog in a spider, has been reported (Damen et al., 2005). Therefore, the role of *slp* homologs in pair-rule pattering in short-germ insects and other arthropods has yet to be determined.

As functional analysis via RNAi becomes available in nondrosophilid insects (Brown et al., 1999b), many noncanonical functions of segmentation genes are being reported at the level of gap and pair-rule genes, suggesting that pair-rule patterning, if functional, is quite different in other insects from *Drosophila* (Bucher and Klingler, 2004; Cerny et al., 2005; Liu and Kaufman, 2005; Mito et al., 2005; Patel et al., 2001). However, ethylmethane sulphonate (EMS) mutagenesis in *Tribolium* identified two phenotypically complementary pair-rule mutants, *scratchy* (*scy*) and *itchy* (*icy*), providing evidence that a pair-rule mechanism plays a role in *Tribolium* segmentation (Maderspacher et al., 1998). Their phenotypes did not suggest obvious *Drosophila* homologs, and a lack of molecular characterization of these mutants has restricted our understanding of pair-rule pattering in this short-germ insect. Recently, in our RNAi analysis of the *Tribolium* homologs of *Drosophila* pair-rule genes, we found that *Tc-prd* and *Tc-slp* RNAi phenocopy the mutant effects of *scy* and *icy*, respectively (Choe et al., 2006). Here we report the roles of *Tc-prd* and *Tc-slp* in *Tribolium* segmentation. Using RNAi to analyze the function of *Tc-prd* and *Tc-slp* revealed that *Tc-prd* is required for odd-numbered segment formation, while *Tc-slp* is required for formation of both odd- and even-numbered segments. *Tc-prd* activates *Tc-en* stripes in odd-numbered parasegments and adjacent *Tc-wg* stripes in even-numbered parasegments. Complementary to *Tc-prd*, the pair-rule function of *Tc-slp* activates *Tc-wg* stripes in odd-numbered parasegments. In addition, it is required as a segment polarity gene to maintain *Tc-wg* stripes. Thus, *prd* functions in the same parasegmental register in *Drosophila* and *Tribolium* whereas the parasegmental register of *slp* function is opposite in one relative to the other. We discuss the implications of these results for the evolution of secondary pair-rule gene functions and the possible use of *prd* and *slp* to study pair-rule patterning in other short-germ arthropods.

Materials and methods

Identification and RT-PCR cloning of Tc-prd and Tc-slp

The previously cloned homeodomain fragment of *Tc-prd* and the forkhead domain fragment of *Tc-slp* (Choe et al., 2006) were used to computationally identify candidate loci in the *Tribolium* genome (http://www.hgsc.bcm.tmc.edu/projects/tribolium/). Initially, each full-length CDS for *Tc-prd* and *Tc-slp* was predicted manually by comparison with protein sequences from *Drosophila* Prd and Slp respectively. The manually predicted full-length CDS sequences were almost identical to the genes computationally predicted (*Tribolium* genome project, HGSC, Baylor college of medicine). A set of primers was designed from the putative 5′ and 3′-UTRs of the predicted *Tribolium* sequences and used to amplify fragments containing full-length *Tc-prd* or *Tc-slp* coding sequences. Total RNA was isolated from 0- to 48-h embryos using Trizol (Invitrogen) and cDNA was synthesized from total RNA template using SuperScript™ III Reverse Transcriptase (Invitrogen). PCR was performed with Takara Ex Taq™ DNA Polymerase (Takara) and the amplicons were cloned into Promega's pGEM®-T Easy Vector (Promega). Sequences were determined on an ABI 3730 DNA Analyzer using BigDye Terminators (Kansas State University DNA Sequencing and Genotyping Facility (http://www.oznet.ksu.edu/pr_dnas/)). The cDNA sequences have been deposited in Genbank under the accession number of DQ414247 for the *Tc-prd* CDS and DQ414248 for the *Tc-slp* CDS.

Parental RNAi and embryo collection

Parental RNAi was performed as described (Bucher et al., 2002) using 500 ng/μl of *Tc-prd* and *Tc-slp* dsRNA to produce severe RNAi effects. 1× Injection buffer or 1 μg/μl of *Tc-ftz* dsRNA was injected as a control and, as previously observed (Choe et al., 2006), did not generate any mutant phenotypes. To analyze the hypomorphic series of RNAi phenotypes, embryos were collected every 48 h for 6 weeks, during which time the observed phenotypes became less and less severe until only wild-type larva were produced. Embryos were incubated at 30°C for 4 days to complete embryogenesis and then placed in 90% lactic acid to assess cuticular effects. For whole-mount in situ hybridization and immunochemistry, 0- to 24-h embryos were collected and fixed by standard protocols.

Whole-mount in situ hybridization and immunochemistry

Whole-mount in situ hybridization was performed as previously described (Brown et al., 1994) with some modifications. To devitellinize eggs and dissect germbands from the yolk, fixed embryos were incubated in 50% xylene and vortexed at high speed for 30 s every 10 min for 1 h. The devitellinized and dissected embryos were immediately used for whole-mount in situ hybridization. Immunochemistry was carried out as described with a 1:5 dilution of mAbs 4D9 (anti-En) or a1:20 dilution of 2B8 (anti-Eve) from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa.

Molecular analysis of itchy and scratchy

Homozygous mutant *icy* and *scy* individuals were identified by visual inspection of the progeny in heterozygous male lines. Genomic DNA was isolated by grinding one larva in 50 μl of squish buffer (Gloor et al., 1993) and incubating it with proteinase K for 1 h at 25°C. 2 μl of lysate from a squished larva was used as template for PCR. To survey for sequence changes in the exon of the candidate loci of the mutants, each exon was amplified from the mutants, cloned and sequenced, as described above. The sequences were aligned with wild-type exon sequences using CLUSTAL W with default parameters (Thompson et al., 1994).

Results

Tribolium paired and sloppy-paired homologues

Homologues of *prd* and *slp* were predicted by BLAST analysis of the *Tribolium* genome. We generated PCR clones containing full-length coding sequences for these genes from wild-type cDNA. Comparison with genomic DNA confirmed the computational prediction and indicated that the *Tc-prd* locus is about 29 kb with 5 exons. The deduced 387 aa protein sequence contains a paired domain and a homeodomain similar to those found in *Drosophila* Prd (Fig. 1A). Tc-Prd does not contain the octapeptide that distinguishes *Drosophila gooseberry* and *gooseberry-neuro*, and the *Schistocerca pairberry* (Davis et al., 2001). There is 84.5% identity within the paired domain and 91.5% within the homeodomain between *Drosophila* and *Tribolium*.

A single *Tc-slp* gene was found by BLAST analysis of the *Tribolium* genome. Similar to *Drosophila*, the *Tc-slp* locus is approximately 1.3 kb and contains a single exon encoding 312 aa. The forkhead domain and two short domains (domain II and III) are highly conserved; the forkhead domain of *Tc-slp* is 83.2% identical to the forkhead domain of *Drosophila slp1*, but 95.3% identical to that of *Drosophila slp2* (Fig. 1C). Additional sequence similarity between *Tc-slp* and *Dm-slp2* is apparent throughout the proteins, including the last 12 residues at the carboxy-terminus.

Expression patterns of Tc-prd

Previously, the expression patterns of Pax group III genes were analyzed in *Tribolium* with a polyclonal antibody that crossreacts with *Drosophila* Prd, Gooseberry and Gooseberry-neuro (Davis et al., 2001). Because the expression domains of these genes are expected to overlap in *Tribolium* segmentation as in *Drosophila*, we used whole-mount in situ hybridization to follow the expression of just *Tc-prd*. Anti-En antibody was used as a marker to determine the register of the *Tc-prd* expression domain. Transcripts of *Tc-prd* first appear in a narrow stripe at about 60% egg length (measured from the posterior pole) during the blastoderm stage (Fig. 2A). This stripe forms in the presumptive mandibular segment, as evidenced by the fact that it overlaps the first Tc-En stripe and extends anteriorly from it (Figs. 2A, B). Similar to the mandibular stripe of *Drosophila prd*, this *Tribolium prd* stripe does not resolve into two secondary stripes (Kilchherr et al., 1986). Immediately following condensation of the germ rudiment, the second *Tc-prd* stripe appears posterior to the first, and the gradient of expression within this broad stripe is strongest at the posterior boundary (Fig. 2C). This primary stripe covers an entire even-numbered parasegment and the Tc-En stripe in the next odd-numbered parasegment. It resolves into two secondary stripes by fading in the center, from posterior to anterior (Fig. 2D). Consequently, two secondary stripes of *Tc-prd* form; the weaker anterior stripe (*Tc-prd* b) corresponds to a Tc-En stripe in an even-numbered parasegment and the stronger posterior stripe (*Tc-prd* a) corresponds to a *Tc-wg* stripe and the adjacent Tc-En stripe in even- and odd-numbered parasegments respectively (Fig. 2E and summarized in Fig. 7A). These secondary stripes fade completely as the embryo develops. Similar to *Drosophila*, Tc-En stripes appear after the secondary *Tc-prd* stripes suggesting a similar role for *Tc-prd* as a regulator of *Tc-en* (Fig. 2E). During subsequent germband growth, additional *Tc-prd* stripes appear in the middle of the growth zone and resolve into two secondary stripes that eventually fade (Figs. 2E–I). This is similar to the dynamics of *Tc-eve* and *Drosophila prd* expression (Brown et al., 1997; Kilchherr et al., 1986; Patel et al., 1994). Therefore, we conclude that *Tc-prd* is expressed in a pair-rule manner. Interestingly, as the germband fully extends, a narrow *Tc-prd* stripe is detected in the posterior region of the germband immediately after the fifteenth Tc-En stripe (arrow in Fig. 2I). Similar to the first stripe observed in the presumptive head region at the blastoderm stage, this final stripe is not pair-rule like. It seems likely that these two *Tc-prd* stripes are regulated differently from the other stripes that are expressed in double segment periodicity during segmentation.

Tc-prd is required for odd-numbered segment formation

To gain further insight into the role of *Tc-prd*, we extended our previous analysis of *Tc-prd*^{RNAi} embryos (Choe et al., 2006). Across a gradient of *Tc-prd*^{RNAi} effects, gnathal and

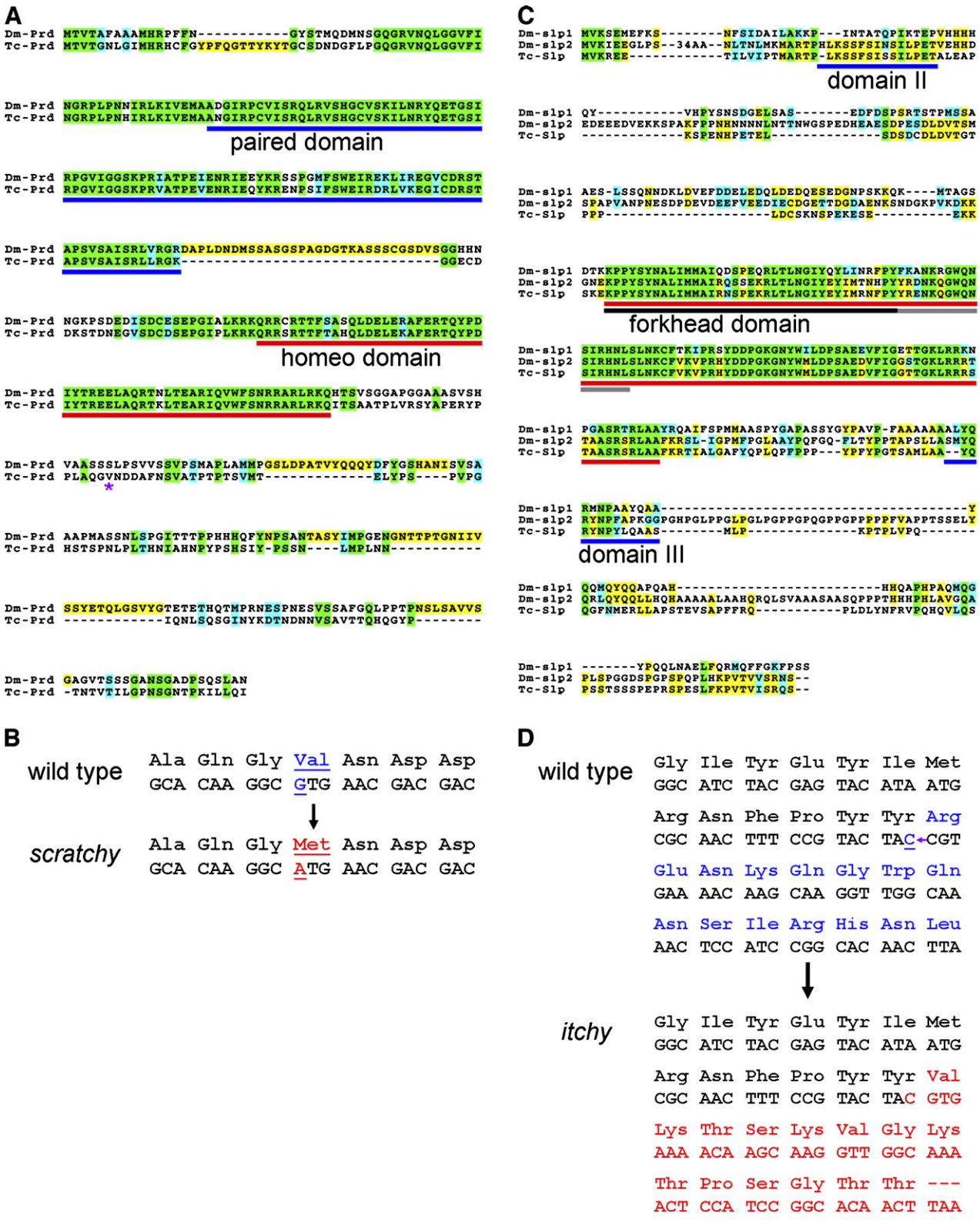

Fig. 1. Molecular characterization of *Tc-prd* and *Tc-slp*, and identification of the mutations in *scy* and *icy*. (A) *Tc-prd* contains two highly conserved domains, a paired domain and a homeodomain. The amino acid substituted in *scy* is marked with an asterisk. (B) *Scy* might be caused by a point mutation in the region following the homeodomain. The point mutation causes substitution of valine for methionine. (C) *Tc-slp* contains the conserved domains II and III (blue lines) as well as a forkhead domain (red line). The truncated forkhead domain in *icy* is underlined with black (same amino acids as wild-type) and gray lines (substituted amino acids). (D) Deletion of a single nucleotide in the forkhead domain caused a shift in the reading frame followed by truncation after 14 amino acids (red) in the *icy* mutant.

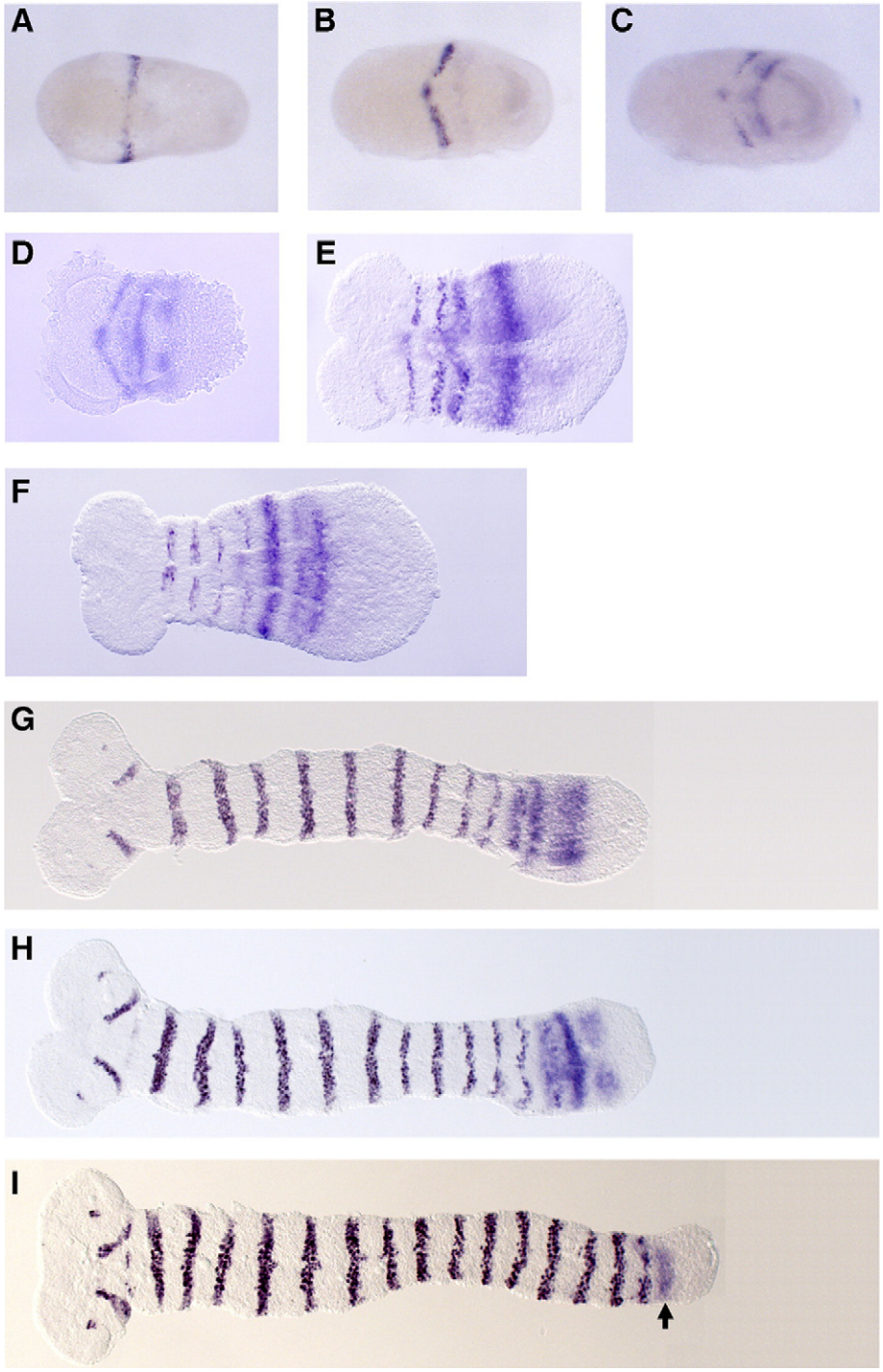

Fig. 2. Expression of *Tc-prd* in *Tribolium* embryos undergoing segmentation. Panels A–C and E–I are stained with *Tc-prd* riboprobe (purple) and Anti-En antibody (punctuate, brown spots). (A) In the blastoderm, a narrow stripe of *Tc-prd* appears coincident with the first Tc-En stripe and extends anteriorly within the presumptive mandibular segment. (B) As the germ rudiment forms, the first *Tc-prd* stripe is restricted to embryonic tissue on the ventral side of the egg. (C) The second *Tc-prd* stripe appears just after the germband forms. Expression in this broad primary stripe is stronger at the posterior edge. (D) In this embryo, the in situ hybridization was performed without the antibody staining to show the second *Tc-prd* stripe resolving into two stripes (*Tc-prd* a and b). The third primary stripe appears posterior to the second. (E) The third *Tc-prd* stripe appears in same manner as the second *Tc-prd* stripe. By this time the first *Tc-prd* stripe has completely faded but En staining is still observed. (F) The second stripe has faded as the fourth stripe appears. (G, H) During germband elongation primary *Tc-prd* stripes appear de novo in the middle of the growth zone, resolve into two secondary stripes as described above and eventually fade. (I) In this fully elongated germband, a narrow *Tc-prd* stripe (arrow) appears just after the fifteenth Tc-En stripe.

thoracic segments always displayed clear pair-rule phenotypes (Figs. 3B, C). However, the series of *Tc-prd*^{RNAi} embryos showed variation in the number of abdominal segments affected (Figs. 3B, C, compare to 3A). Most *Tc-prd*^{RNAi} embryos (90.2%) were strongly affected and displayed complete pair-rule phenotypes containing only 4 or 5 abdominal segments

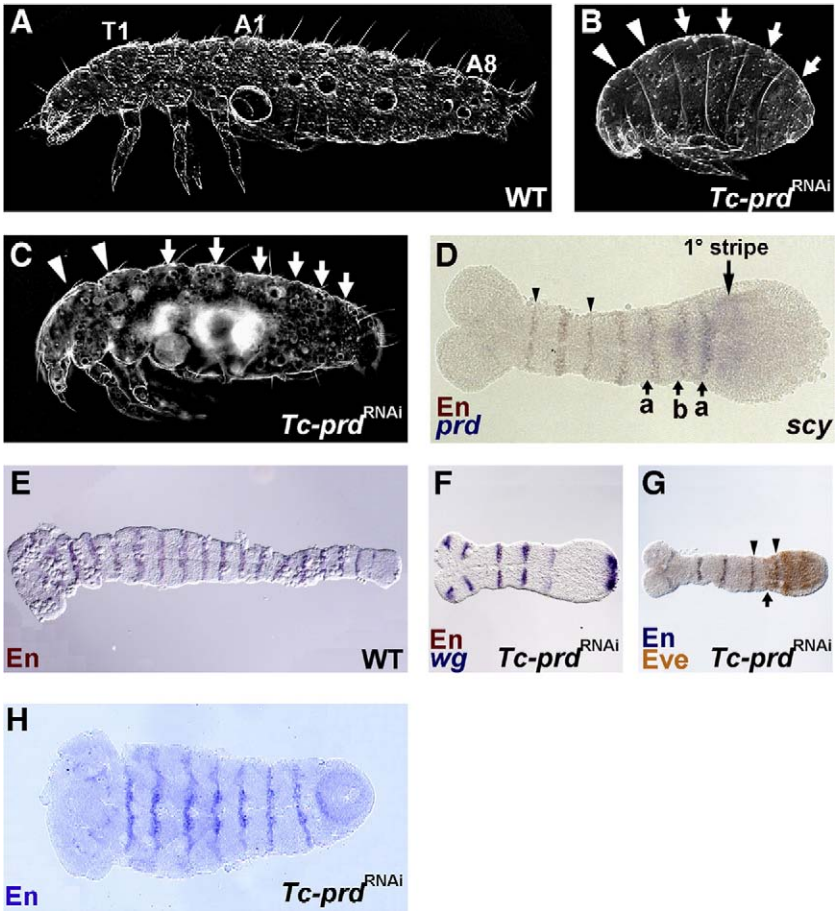

Fig. 3. Cuticle preparations and germband defects in *Tc-prd*^{RNAi} or *scy*. (A–C) Cuticle preparations. (D–H) Germbands undergoing segmentation. (A) Lateral view of wild-type first instar larval cuticle with head, three thoracic segments (T1–T3), eight abdominal segments (A1–A8) and telson. (B, C) Cuticular phenotypes of *Tc-prd*^{RNAi}. Thoracic segments, arrow heads; abdominal segments, arrows. (B) This severely effected *Tc-prd*^{RNAi} embryo still contains Mx, T1, T3 and four abdominal segments. (C) This less severely effected *Tc-prd*^{RNAi} individual contains Mx, T1, T3 and six abdominal segments. (D) Elongating germband of *scy* embryo stained with anti-En antibody (punctuate, brown spots) and *Tc-prd* (purple). The defective odd-numbered En stripes are marked with arrow heads whereas the normal *Tc-prd* stripes are marked with arrows. (E) Fully elongated wild-type germband stained with anti-En antibody. In this wild-type germband, a total of 16 Tc-En stripes form. (F) Elongating germband of *Tc-prd*^{RNAi} embryo stained with anti-En antibody (punctuate, brown spots) and *Tc-wg* (purple). Every other Tc-En and its adjacent *Tc-wg* stripe are gone. (G) Elongating germband of *Tc-prd*^{RNAi} embryo stained with anti-En (punctate, dark blue spots) and anti-Eve antibodies (punctuate, brown spots). In this germband, odd-numbered Tc-En stripes, which coincide with Tc-Eve a stripes (arrow) are missing, whereas even-numbered Tc-En stripes which coincide with Tc-Eve b stripes (arrow head) form normally. (H) *Tc-prd*^{RNAi} germband stained with anti-En antibody after germband retraction. 7 Total Tc-En stripes are expressed revealing a classic pair-rule phenotype. T, thoracic segment; A, abdominal segment. Anterior is to the left.

(Fig. 3B) while weak *Tc-prd*^{RNAi} embryos (8.7%), showed deletion of 3 or fewer abdominal segments (Fig. 3C), which is similar to the common phenotypes described in the *scy* mutant (Maderspacher et al., 1998).

To determine the register of segmental deletions, we followed the expression of the segment polarity genes *Tc-en* and *Tc-wg* in *Tc-prd*^{RNAi} embryos. In contrast to *scy* in which every other *Tc-en* and its adjacent *Tc-wg* stripes were weakly initiated with normal initiation of the alternate *Tc-en* and *Tc-wg* stripes (Maderspacher et al., 1998), every other *Tc-en* and its adjacent *Tc-wg* stripe were not activated at all in the *Tc-prd*^{RNAi} embryos (Figs. 3F, H, compare to E). Furthermore, double staining *Tc-prd*^{RNAi} embryos for Tc-Eve and Tc-En showed that Tc-En stripes normally expressed in the odd-numbered parasegments are missing (Fig. 3G). Thus, *Tc-prd* is required for formation of all odd-numbered segments through activation of *Tc-en* stripes in odd-numbered parasegments and the adjacent *Tc-wg* stripes in even-numbered parasegments (summarized in Fig. 7B). This function of *Tc-prd* is consistent with the alternating intensity of the secondary segmental stripes of *Tc-prd* in which the strong secondary stripes (*Tc-prd* a) overlap the Tc-En stripe in odd-numbered parasegments and the adjacent *Tc-wg* stripe in even-numbered parasegments while the weak stripes (*Tc-prd* b) overlap the Tc-En stripes in even-numbered parasegments (Figs. 7A, B). Similarly in *Drosophila*, *prd* functions as an activator of *en* stripes in odd-numbered parasegments and their adjacent *wg* stripes in even-numbered parasegments (Fig. 7B), and null alleles of *prd* cause a complete pair-rule phenotype where every odd-numbered segment is deleted (Ingham et al., 1988). The conserved expression and function of *prd* in *Drosophila* and *Tribolium* suggests that their common ancestor contained a *prd* gene with a similar pair-rule function in segmentation.

Expression patterns of Tc-slp

In contrast to the extensive studies of Pax group III gene expression patterns in various insects and basal arthropods, the expression pattern of *slp* has been reported only for *Drosophila* and the spider *Cupiennius salei* (Damen et al., 2005; Grossniklaus et al., 1992). In *Drosophila*, *slp1* is initiated in the presumptive head region in a broad, gap-like pattern where it is required for segment formation. Soon thereafter, primary *slp1* stripes appear in every even-numbered parasegment. Then secondary *slp1* stripes intercalate between the primary stripes, resulting in segmental expression of *slp1*. *slp2* is expressed in the same trunk domain as *slp1* with a temporal delay, and it is not expressed in the presumptive head. In the spider, *slp* is expressed with a single segment periodicity instead of double segment periodicity.

To understand possible segmental functions of *Tc-slp*, we analyzed its expression pattern. During the blastoderm stage, a broad stripe of *Tc-slp* transcripts appears at about 70% egg length from the posterior pole (Fig. 4A). Soon thereafter this stripe is limited ventrally in the presumptive head lobes of the future germ rudiment (Fig. 4B), in the regions that give rise to the antennae (Fig. 4J). Before the germ rudiment condenses, a new *Tc-slp* stripe appears in the blastoderm (arrow head in Fig. 4C). Double staining with anti-En antibody indicates that this second stripe is expressed in the presumptive mandibular segment (Fig. 4E). Just after the germband forms, a narrow *Tc-slp* stripe appears in the presumptive maxillary segment (arrow head in Fig. 4D). Then a strong stripe (arrow heads in Figs. 4E, F) in the first thoracic segment appears prior to a weak narrower stripe in the labial segment (arrow Fig. 4F). During germband elongation, pairs of *Tc-slp* stripes appear in the anterior region of the growth zone (Figs. 4G–K). The anterior stripe (arrows in Figs. 4G–K) is narrower and weaker than the posterior stripe (arrow heads in Figs. 4G–K). As they develop, each *Tc-slp* stripe overlaps the anterior row of cells in a Tc-En stripe (Figs. 4G–J). To differentiate these stripes, we defined the stronger posterior stripe as *Tc-slp* a, most of which is in an odd-numbered parasegment, and the anterior stripe as *Tc-slp* b, most of which is in an even-numbered parasegment. The dynamics of the *Tc-slp* expression pattern is summarized in Fig. 7A. Typical of a pair-rule gene, *Tc-slp* stripes a and b define two segments at once during germband elongation. The difference in intensity between these two stripes suggests they may have different functions in segmentation. All *Tribolium* pair-rule genes reported to date show transient expression patterns; their expression initiates in the growth zone and fades away in the elongating germband (Brown et al., 1994; Brown et al., 1997; Patel et al., 1994; Sommer and Tautz, 1993). However, *Tc-slp* expression is not transient, but is maintained in a segmental pattern until the germband is fully elongated, which is similar to the expression of segment polarity genes. This is not unexpected, since *slp* genes continue to be expressed as the *Drosophila* germband develops (Grossniklaus et al., 1992). In summary, *Tc-slp* expression is similar to that of *Drosophila slp1* and *2* in that the expression pattern initiates in a pair-rule pattern and then remains during germband elongation similar to a segment polarity gene. *Tc-slp* expression is different in that a pair of stripes initiates simultaneously and the register of strong and weak stripes is the opposite of *slp* stripes in *Drosophila*.

Tc-slp is required for gnathal segmentation, formation of even-numbered segments and maintenance of the odd-numbered segments in the trunk

We analyzed a graded series of *Tc-slp*^{RNAi} embryos to better understand the function of *Tc-slp* during segmentation. First, all the gnathal segments (mandibular, maxillary, and labial), are defective across the entire gradient of *Tc-slp*^{RNAi} embryos (Figs. 5B, C, compare to 5A) suggesting that *Tc-slp* performs a gap-like function in the gnathum. In *Drosophila*, *slp1* functions as a head gap gene; a null mutant of *slp1* causes defects in mandibular and pregnathal segments (Grossniklaus et al., 1994). However, *Tc-slp* did not show any evidence of a gap gene-like expression pattern. Instead, it is initiated as narrow stripes at the blastoderm and early germband stages (Figs. 4B–F). Thus, individual stripes in each segment, rather than gap gene-like expression of *Tc-slp* appear to be required for gnathal segmentation. In addition, *Tc-slp*^{RNAi} displayed a range of phenotypes in the abdominal segments (Figs. 5B, C, compare to A).

The most severe *Tc-slp*^{RNAi} embryos (8.3%) displayed a compact segmental phenotype with 4 asymmetrically incomplete segments (Fig. 5B; see 4 segments (white dots) on one side and 2 broad segments (white arrow heads) on the other side). However, most of the *Tc-slp*^{RNAi} embryos (91.7%) displayed a classical pair-rule phenotype in which T1, T3 and only 4 or 5 abdominal segments were missing (Fig. 5C).

To molecularly identify the defective segments, we followed the expression of the segment polarity genes *Tc-en* and *Tc-wg* in *Tc-slp*^{RNAi} embryos. In wild-type embryos, *Tc-en* and the adjacent *Tc-wg* stripes are initiated by pair-rule genes and then maintained by the *Tc-en*, *Tc-hedgehog*, and *Tc-wg* circuit during germband elongation (Farzana and Brown, unpublished data). In most *Tc-slp*^{RNAi} embryos at the elongated germband stage, all the gnathal stripes as well as every other stripe of Tc-En and *Tc-wg* were missing, supporting the combined head gap and pair-rule phenotypes observed in *Tc-slp*^{RNAi} cuticles. However, analysis of younger embryos revealed that *Tc-slp*^{RNAi} completely abolished the initiation of a *Tc-wg* stripe but not the adjacent Tc-En stripe (Fig. 5G, compare to E). And although it is initiated, Tc-En expression in these defective segments was not maintained, probably due to the absence of neighboring *Tc-wg* expression. Double staining with anti-Eve and anti-En antibodies to determine the register of the remaining Tc-En stripes demonstrated that the defective Tc-En and *Tc-wg* stripes are in even-numbered and adjacent odd-numbered parasegments respectively (Fig. 5H). Thus, in the trunk the missing Tc-En and *Tc-wg* stripes correspond to T1, T3 and the even-numbered abdominal segments (summarized in Fig. 7B). Taken together, these results indicate that *Tc-slp* a, which is expressed in odd-numbered parasegments, is required in there for the activation of *Tc-wg* stripes as well as for the maintenance of the adjacent Tc-En stripes (in even-numbered parasegments)

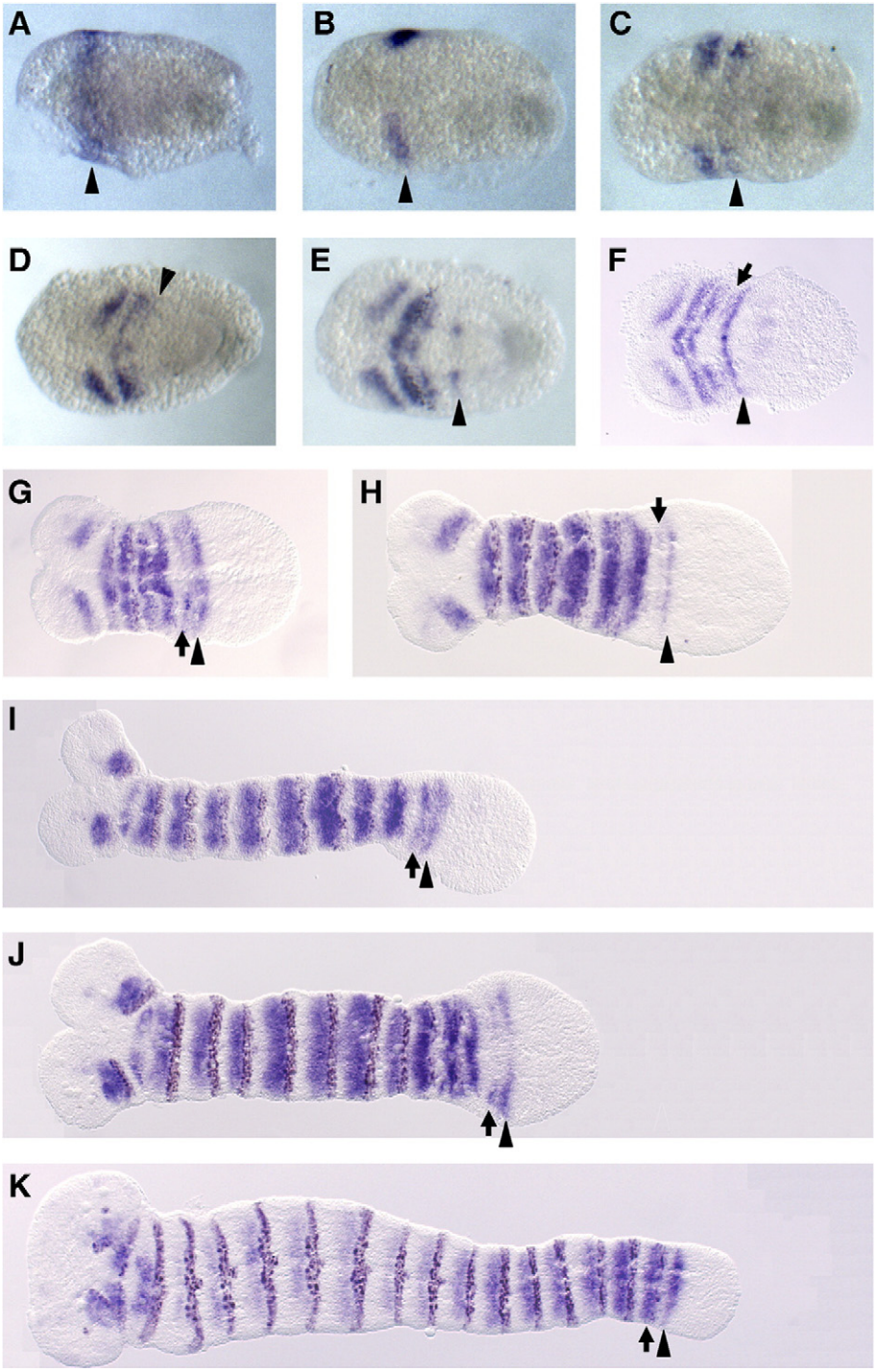

Fig. 4. Expression of *Tc-slp* in *Tribolium* embryos undergoing segmentation. (A–D, F) Stained with *Tc-slp* riboprobe (purple). (E, G–K) Stained with *Tc-slp* riboprobe (purple) and Anti-En antibody (punctuate, brown spots). (G–K) Primary *Tc-slp* stripes, arrow head; Secondary *Tc-slp* stripes, arrow. (A–D) Blastoderm stage. (E–K) Germband stages. (A) The first *Tc-slp* stripe (arrow head) appears de novo in the anterior region of the embryo (future head lobes). (B) This stripe (arrow head) is split by the mesoderm at the ventral midline. (C) The second *Tc-slp* stripe (arrow head) appears first in the ectoderm and then in the mesoderm (D). The third *Tc-slp* stripe (arrow head in panel D) is initially narrower and weaker than the second stripe. (E) The second *Tc-slp* stripe is expressed in the mandibular segment as evidenced by its position relative to the first Tc-En stripe formed at the posterior border of mandibular segment. In addition, the fifth *Tc-slp* stripe (arrow head) appears as two spots flanking the mesoderm. (F) A narrow and weak fourth *Tc-slp* stripe (arrow) appears anterior to the fifth stripe (arrow head). (G) A pair of *Tc-slp* stripes (arrow and arrow head) appears posterior to the previous *Tc-slp* stripes. The anterior stripe of the pair (*Tc-slp* b; arrow) is weak while the posterior one (*Tc-slp* a; arrow head) is strong. (H–K) The next pair of *Tc-slp* stripes (arrow and arrow head) forms posterior to the previous pair. *Tc-slp* stripes do not fade, rather they become broader as the segments develop. Anterior is to the left.

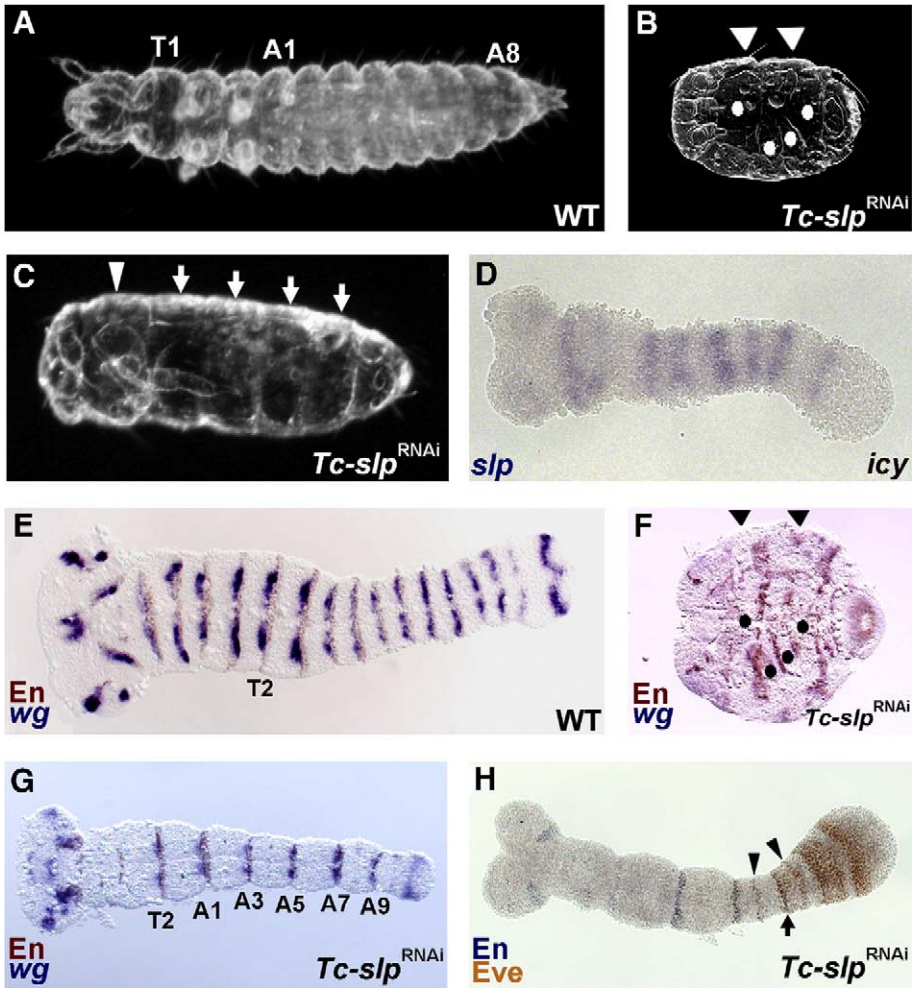

Fig. 5. Cuticle preparations and germband defects in *Tc-slp*^{RNAi} or *icy*. (A–C) Cuticle preparations. (D–H) Germbands undergoing segmentation. (A) Ventral view of wild-type first instar larval cuticle with head, three thoracic segments (T1–T3), eight abdominal segments (A1–A8) and telson. The head contains mandibles, as well as maxillary and labial palps. (B, C) Cuticular phenotypes of *Tc-slp*^{RNAi} embryos. (B) The most severe phenotype of *Tc-slp*^{RNAi} produces embryos with two giant segments on one side (arrow heads) and four segments on the other (white dots). (C)The intermediate phenotype of *Tc-slp*^{RNAi} produces embryos containing T2 (arrow head) and four abdominal segments (arrows) but does not have any gnathal segments. (D) Elongating germband of *icy* embryo stained with *Tc-slp* (purple). Segmental expression in the trunk is weak (compare to Fig 4I) whereas the expression in the gnathal is irregular and almost abolished. (E–G) Wild-type and *Tc-slp*^{RNAi} embryos stained with anti-En antibody (punctuate, brown spots) and *Tc-wg* in situ (purple) (E) In this wild-type germband, 16 Tc-En and *Tc-wg* stripes (purple) form. (F) In this representative of the most severe *Tc-slp*^{RNAi} germbands, two wider than normal Tc-En stripes (arrow head) and several incomplete Tc-En stripes (arrow) remain after germband retraction. The pattern of Tc-En stripes in this germband is almost identical to the segmental grooves in panel B. (G) In this elongating *Tc-slp*^{RNAi} germband, every other set of Tc-En and *Tc-wg* stripes is defective, and the anterior *Tc-wg* stripes have faded while the new posterior *Tc-wg* stripes formed normally. (H) Elongating germband of *Tc-slp*^{RNAi} embryo stained with anti-En (punctuate, dark blue spots) and anti-Eve antibodies (punctuate, brown spots). In this germband, even-numbered Tc-En stripes, which were coexpressed with Tc-Eve b stripes (arrow head) are missing whereas odd-numbered Tc-En stripes coincident with Tc-Eve a stripes (arrow) form normally. T, thoracic segment; A, abdominal segment. Anterior is to the left.

leading to the formation of even-numbered segments (Figs. 7A, B). In *Drosophila*, *slp* functions as a pair-rule gene in combination with *prd*, to activate *wg* stripes in even-numbered parasegments (Fig. 7B), which eventually leads to the formation of odd-numbered segments (Cadigan et al., 1994b; Coulter and Wieschaus, 1988; Ingham et al., 1988). Thus, the primary requirement for *slp* has evolved differently in *Drosophila* and *Tribolium*.

Interestingly, in addition to the loss of *Tc-wg* stripes in odd-numbered parasegments and the neighboring Tc-En stripes in even-numbered parasegments, as described above, some more severely affected *Tc-slp*^{RNAi} embryos showed additional loss of the *Tc-wg* stripes that had formed normally in even-numbered parasegments. Although initiated, they were not properly maintained and began fading before the germband fully extended (compare the T2 *Tc-wg* stripes in Figs. 5G and E) implying that *Tc-slp* b, which is expressed in even-numbered parasegments, is required to maintain *Tc-wg* stripes in these parasegments. Furthermore, these decay dynamics provide support for the most severe *Tc-slp*^{RNAi} phenotypes in that the Tc-En stripes, which are initiated normally in odd-numbered parasegments, were not maintained sufficiently (due to the loss of *Tc-wg* stripes in adjacent even-numbered parasegments) to form segmental grooves (Fig. 5F, compare to B). Thus, the most severe *Tc-slp*^{RNAi} phenotypes appear to be caused by the combination of failing to initiate even-numbered segments and

failing to maintain odd-numbered segments. In summary, we conclude that the *Tc-slp* a stripes are required for the formation of even-numbered segments through the activation of *Tc-wg* stripes in odd-numbered parasegments. Later, *Tc-slp* functions as a segment polarity gene to maintain *Tc-wg* stripes in even-numbered parasegments (*Tc-slp* b) and most likely all parasegments (*Tc-slp* a and b) (Figs. 7A, B). In *Drosophila*, segmentally expressed secondary (segment polarity) *slp* stripes are required to maintain *wg* stripes, and *slp* null individuals display a pair-rule phenotype in the thorax (T1-T2 and T3-A1 fusions) and a *wg*-class segment polarity phenotype in the abdomen (lawn of denticles) (Cadigan et al., 1994b). Thus, although flies require *slp* function in a segmental register opposite that in beetles for pair-rule patterning, the overall requirement is similar, in that it is required early for the initiation of every other segment and later for the maintenance of the remaining segments, if not all segments.

Segmental identity is not altered by the loss of Tc-prd or Tc-slp

Homeotic transformation has been reported for *Tribolium* gap gene mutants or in gap gene RNAi embryos (Bucher and Klingler, 2004; Cerny et al., 2005). Because it has been speculated that the homeotic defects are mediated by pair-rule genes (Cerny et al., 2005), we asked whether *Tc-prd* and *Tc-slp* are involved in determining segmental identity as well as segment formation. Cuticular phenotypes of *Tc-prd*^{RNAi} or *Tc-slp*^{RNAi} embryos did not show any homeotic defects implying that these pair-rule genes are not involved in the regulation of homeotic genes (Figs. 3B, C, 5B, C). In *Tribolium*, *Deformed* (*Dfd*) is expressed in the mandibular and maxillary segments (Brown et al., 1999a), *Sex combs reduced* in the posterior maxillary and labial segments (Curtis et al., 2001) and *Ultrabithorax* from T2 through the abdominal segments (Bennett et al., 1999). We performed in situ hybridization with these three homeotic genes, as markers of segmental identity in the *Tc-prd*^{RNAi} or *Tc-slp*^{RNAi} embryos. Consistent with the cuticular phenotypes, these homeotic genes were expressed normally in the *Tc-prd*^{RNAi} or *Tc-slp*^{RNAi} embryos (data not shown) except for *Dfd* in *Tc-slp*^{RNAi} embryos where its expression was limited to a narrow region near the head lobes (Fig. 6C, compare to A, B). In *Drosophila*, not all pair-rule genes are involved in determining segmental identity (Ingham and Martinez-Arias, 1986); *ftz* is required for the regulation of homeotic genes but *prd* is not. Even though we cannot completely exclude the possibility that other pair-rule genes are involved in the determination of segmental identity, it appears that neither *Tc-prd* nor *Tc-slp* functions to determine segmental identity.

Scratchy and itchy are potential Tc-prd and Tc-slp mutants, respectively

Tc-prd^{RNAi} cuticles have maxillary palps, two pairs of legs and 4 abdominal segments; they are missing odd-numbered segments. *Tc-slp*^{RNAi} cuticles typically contain a single pair of legs and 4 abdominal segments; they lack all gnathal segments

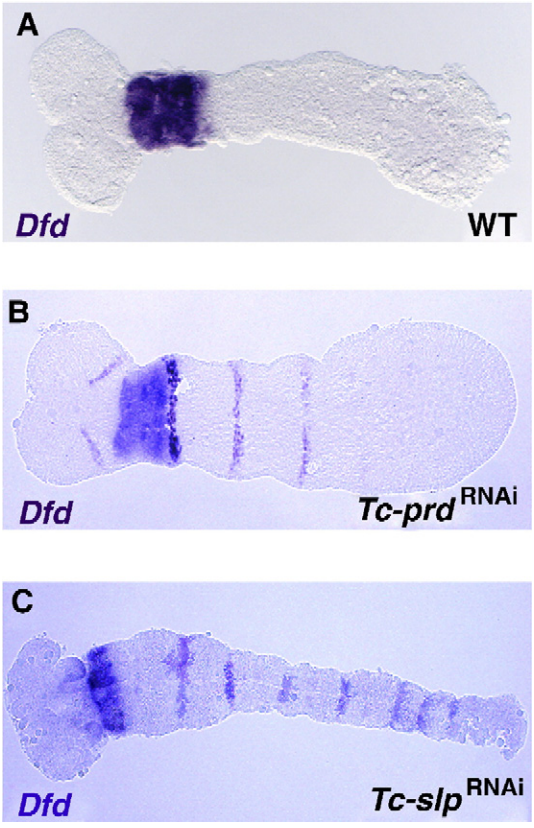

Fig. 6. *Tc-Dfd* expression in *Tribolium* germband embryos. (A) *Tc-Dfd* mRNA (purple) is expressed in the mandibular and maxillary segments in this wild-type germband. (B–C) Expression of *Tc-Dfd* mRNA (purple) and Tc-En protein (punctuate, brown spots) in *Tc-prd*^{RNAi} and *Tc-slp*^{RNAi} germband embryos. (B) In this germband, *Tc-Dfd* expression overlaps the first even-numbered Tc-En stripe (maxillary stripe) in a domain that is two-segment wide but lacking the mandibular Tc-En stripe. (C) *Tc-Dfd* is expressed in a narrower more anterior domain. Note the two-segment wide spacing between Tc-En stripes in the trunk and anterior abdomen. Anterior is to the left.

and even-numbered segments in the trunk. Interestingly, these RNAi effects phenocopy the mutant phenotypes of two complementary, EMS induced mutations in *Tribolium*, *scy* and *icy* (Maderspacher et al., 1998). In the *scy* mutant, we found a point mutation in exon 4 of *Tc-prd*, which causes a valine to methionine change after the homeodomain (Fig. 1B). Alignment of the protein sequences indicated that this region is not highly conserved between *Drosophila* and *Tribolium* (asterisk in Fig. 1A), making it difficult to imagine how this missense mutation may cause the *scy* phenotype. However, two *Drosophila prd* alleles, *prd*X3 and *prd*IIN indicate that this region, immediately after the homeodomain, is important for the in vivo function of Prd (Bertuccioli et al., 1996). *Tc-prd* transcripts are expressed in *scy* mutant embryos, indicating that the mutant phenotype is more likely to be due to the production of a non-functional protein than a regulatory defect (Fig. 3D). Finally, the highly variable phenotype described for *scy* (Maderspacher et al., 1998) is indicative of a hypomorphic mutant. Intriguingly, *Tc-prd*^{RNAi} produces the same range of phenotypes. Thus, the *scy* mutant might be a hypomorphic mutant of *Tc-prd* that is caused by the amino acid substitution in the exon 4 of *Tc-prd* locus.

In comparing the sequence of the *Tc-slp* locus in the *icy* mutant with that of wild-type (GA-1), we detected a single nucleotide deletion in the region encoding the forkhead domain (Fig. 1D). This deletion alters the reading frame and causes truncation about half-way through the forkhead domain (53/107 aa). Considering the importance of this domain to Slp as a transcription factor, it is highly likely that this truncation within the forkhead domain causes the mutant phenotype. Furthermore, we also found that transcripts of *Tc-slp* are expressed in normal segmental pattern with decreased intensity in the trunk whereas the expression is irregular and almost abolished in the gnathal region in the presumptive *icy* embryos (Fig. 5D) indicative of nonsense mediated degradation of the *Tc-slp* transcripts. Therefore, we suggest that the *icy* mutant might be an allele of *Tc-slp* that is caused by the truncation of the forkhead domain in the *Tc-slp*. EMS usually causes deletion of several nucleotides (Anderson, 1995) rather than deletion of a single nucleotide. However, we observed the same nucleotide deletion in six *icy* individuals. Truncation within an essential domain of a transcription factor is expected to produce a null phenotype. However, the *icy* produces a range of phenotypes, none of which are as severe as the most severe class of *Tc-slp*^{RNAi} embryos. Even though the truncation of the forkhead domain of *Tc-slp* and the decreased amounts of *Tc-slp* transcripts in the *icy* mutant, suggest that *icy* might be a *Tc-slp* mutant, we cannot conclude that *icy* is a *Tc-slp* mutant with certainty. Additional evidence such as positional map data or other alleles for complementation tests are required to confirm the identity of *scy* and *icy* mutants as alleles of *Tc-prd* and *Tc-slp*, respectively.

Discussion

We analyzed the expression and function of the secondary pair-rule genes *prd* and *slp* in *Tribolium*. Our RNAi analysis of *Tc-prd* and *Tc-slp* revealed conserved and divergent aspects of these secondary pair-rule genes relative to the function of their *Drosophila* homologs. The function of *prd* is mainly conserved between the two insects while *slp* displays some divergent as well as conserved functions in *Drosophila* and *Tribolium* segmentation. In addition, we discuss the possible evolution of their roles in the lineages of *Drosophila* and *Tribolium*.

The first stripe of *Tc-prd* expression is observed in the presumptive mandible at the blastoderm stage and seven successive stripes are formed near the middle of the growth zone as the germband elongates. Expression in the mandibular stripe is uniform while expression in the successive stripes appears in a gradient that is strongest posteriorly. Each of these stripes splits into two segmental stripes overlapping Tc-En expression and they eventually fade. In *Tc-prd*^{RNAi} embryos odd-numbered Tc-En stripes fail to initiate and the resulting cuticles displayed a typical pair-rule mutant phenotype in which odd-numbered segments are missing.

The first stripe of *Tc-slp* expression appears near the anterior end of the egg and is quickly restricted to the antennal region of the head lobes. The second and third stripes appear in the presumptive mandibular and maxillary segments of the blastoderm. A weak stripe appears in the labial segment after a stronger stripe has formed in T1. As the germband elongates, additional stripes of *slp* are added in pairs, in which the anterior stripe is weaker than the posterior one. These develop into broad segmental stripes of expression that are maintained during germband elongation. In *Tc-slp*^{RNAi} embryos the even-numbered Tc-En stripes are initiated but not maintained. In addition, in the most severe *Tc-slp*^{RNAi} embryos, odd-numbered Tc-En stripes fade later, during germband retraction. Interestingly, *Tc-slp*^{RNAi} cuticles displayed a range of phenotypes from typical pair-rule to severe segment polarity phenotypes, reminiscent of the mixed pair-rule and segment polarity phenotypes described for *Drosophila slp* null mutants.

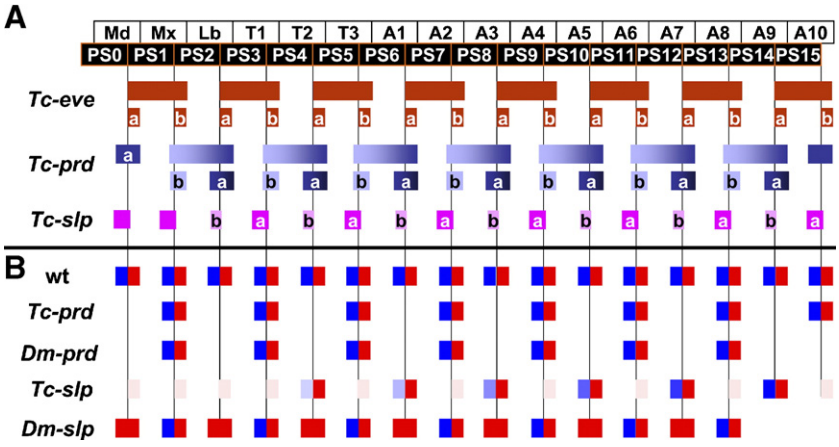

Fig. 7. Summary of secondary pair-rule gene expression relative to other segmentation genes in *Tribolium* and the effects of secondary pair-rule gene mutations or RNAi on the expression of *en* and *wg* in *Drosophila* and *Tribolium*. (A) Pair-rule (upper) and segment polarity (lower) expression domains of *Tc-eve* (brown), *Tc-prd* (dark blue), and *Tc-slp* (pink) in wild-type embryos. Stronger segment polarity stripes are marked with "a" whereas weaker stripes are marked with "b". (B) Expression pattern of *wg* (blue) and *en* (red) in *Tc-prd*^{RNAi} and *Tc-slp*^{RNAi} embryos in addition to stage 9 *Drosophila prd* and *slp*s null mutant embryos. Light red indicates *en* stripes that were weakly initiated but not maintained sufficiently to form segmental grooves during the segmentation. Light blue indicates *wg* stripes that were initiated normally but not maintained during germband elongation.

Functions of prd and slp in segmentation that are conserved between Drosophila and Tribolium

In *Drosophila*, pair-rule genes identified by mutation were named to reflect their phenotypes (Nusslein-Volhard and Wieschaus, 1980). Subsequent molecular characterization of pair-rule genes uncovered expression patterns consistent with the mutant phenotypes, except for *odd-paired* (*opa*), which is expressed ubiquitously but correlated with a pair-rule mutant phenotype (Benedyk et al., 1994). When homologs of *Drosophila* pair-rule genes were shown to have pair-rule expression patterns in certain other insects and basal arthropods, but functional analysis was not available, it was reasonable to speculate that these homologs would have similar functions and thus produce similar loss of function pair-rule phenotypes. However, the systematic functional analysis of *Tribolium* homologs of *Drosophila* pair-rule genes by RNAi revealed that most of them generated phenotypes dramatically different from the pair-rule phenotypes described in *Drosophila*, or no segmental phenotypes, which are not easily explained by their pair-rule expression patterns (Choe et al., 2006). Our analysis indicates that *Tc-prd* and *Tc-slp* RNAi generate a range of phenotypes that include classic pair-rule phenotypes. Furthermore, they are similar to typical *Drosophila* pair-rule genes in that their expression patterns correlate with their mutant phenotypes. For example, the primary stripes of *prd* are expressed between the posterior end of odd-numbered parasegments to the anterior end of next odd-numbered parasegments in both *Drosophila* and *Tribolium*. Interestingly, in *Tribolium*, expression in these primary stripes is stronger toward the posterior edge of each stripe (Fig. 7A), but no such gradient of expression is described for *Drosophila* (Kilchherr et al., 1986). In both insects, the primary stripes split into two secondary stripes. In *Tribolium* the posterior stripe is stronger, but in *Drosophila* they appear to be of equal intensity. In both insects, the secondary stripes coexpressed with En in odd-numbered parasegments are required for segment boundary formation (Ingham et al., 1988). Considering that many homologs of *Drosophila* pair-rule genes show diverse expression patterns or functions in other short-germ insects, it is noteworthy that the expression pattern and function of *prd* are conserved between *Drosophila* and *Tribolium* and suggests that the same expression pattern and function of *prd* was most likely shared by their common ancestor.

Complementary to *Tc-prd*, *Tc-slp* is required as a pair-rule gene for the formation of even-numbered segments and as a segment polarity gene for the maintenance of odd-numbered segments (if not all segments). The segmental stripes of *Tc-slp* are expressed in the posterior region of each parasegment and slightly overlap the Tc-En stripe in the adjacent parasegment (Fig. 7A). *Tc-slp* is similar to *Drosophila slp* (Grossniklaus et al., 1992) in that both are required as pair-rule genes for the activation of alternate *wg* stripes and as segment polarity genes for the maintenance of the remaining *wg* stripes. The more intensely staining *Tc-slp* a stripes, are required for the activation of all gnathal *Tc-wg* stripes and alternate *Tc-wg* stripes in trunk, while the weaker *Tc-slp* b stripes, are required for the maintenance of the remaining *Tc-wg* stripes. Thus, it appears that the function of *slp*, to activate or maintain *wg* expression is conserved between *Drosophila* and *Tribolium*. However, in contrast to *prd* which is required in the same parasegmental register between *Drosophila* and *Tribolium*, *slp* is required in opposite parasegmental registers at the level of pair-rule patterning in *Drosophila* and *Tribolium*. Pair-rule function of *Dm-slp* is required in addition to *Dm-prd* for the activation of *wg* stripes in even-numbered parasegments, while in odd-numbered parasegments, it is required as a segment polarity gene for the maintenance of *wg* stripes that were activated by *Dm-opa* (Benedyk et al., 1994; Cadigan et al., 1994b; Ingham et al., 1988). In contrast, *Tc-slp* functions early as a pair-rule gene to activate *Tc-wg* stripes in odd-numbered parasegments, and later as a segment polarity gene in the maintenance of *Tc-wg* stripes that were initiated normally in even-numbered parasegments. Taken together, our data suggest that the function of *slp* as a pair-rule gene to activate *wg* or as a segment polarity gene to maintain *wg* has been conserved between *Drosophila* and *Tribolium* but that the parasegmental register of *slp* as a pair-rule gene has evolved differently in these two lineages.

Evolution of the role of slp in the network of pair-rule genes in Drosophila and Tribolium

The fact that *prd* is required in the same parasegmental register, while *slp* as a pair-rule gene is required in opposite parasegmental registers in *Drosophila* and *Tribolium* reveals an unprecedented flexibility in the pair-rule mechanism and

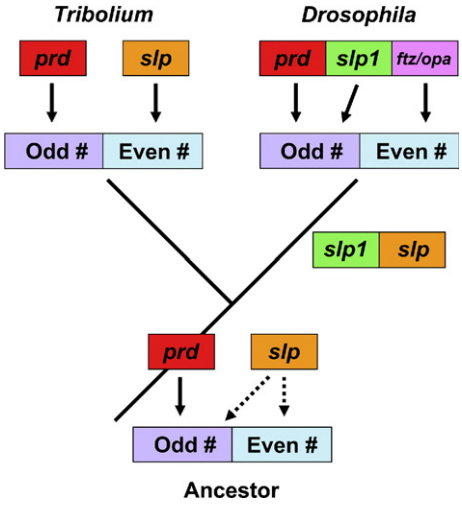

Fig. 8. Comparison of secondary pair-rule gene functions in *Drosophila* and *Tribolium* in an evolutionary context. Across the top of the diagram, the known present pair-rule functions of secondary pair-rule genes in the formation of odd- and even-numbered segments. In *Tribolium*, *Tc-prd* is required in odd-numbered segments and *Tc-slp* is required in even-numbered segments while *Tc-ftz* and *Tc-opa* do not have pair-rule functions. In *Drosophila prd* and *slp1* are required in odd-numbered segments while *ftz* and *opa* are required in even-numbered segments. The segment polarity function of *slp* is not considered in this figure. At the bottom of the diagram, the putative ancestral functions of *prd* and *slp* are shown. It is not yet clear whether *ftz* and *opa* were co-opted in the *Drosophila* lineage or lost in the *Tribolium* lineage.

suggests that the roles of *prd* and *slp* in the pair-rule gene network evolved differently in these insects. Since the parasegmental register for *prd* is conserved in *Drosophila* and *Tribolium* it is likely to be an ancestral feature. In contrast, the different parasegmental register for *slp* suggests the function of *slp* in either *Drosophila*, *Tribolium*, or both is derived. Although it is impossible to determine with certainty the ancestral state of *slp* function when comparing only two species, there are several lines of evidence discussed below that suggest *Tribolium* might more closely resemble the ancestral state.

Considering the highly derived nature of *Drosophila* development, it has often been implied that insects like *Tribolium*, which display more general modes of development, represent ancestral modes of molecular mechanisms as well. In contrast to *Drosophila*, all other nondrosophilid insects and basally branching arthropods examined so far have only one *slp*, whose sequence is more similar to *Dm-slp2* than to *Dm-slp1* (Damen et al., 2005). Thus, it appears that *slp* was duplicated in the lineage leading to *Drosophila* and the sequence of *Dm-slp1* has diverged considerably from the other *slp* genes. However, despite their identical expression patterns, *Dm-slp1,* not *Dm-slp2*, functions as a pair-rule gene in *Drosophila* segmentation (Cadigan et al., 1994a). Later, *Dm-slp2* functions redundantly as a segment polarity gene. We suggest that duplication and subsequent divergence of the *slp* genes are correlated with the differential function of *slp* genes in *Drosophila* and likely contributed to the evolution of the role of *slp* in the *Drosophila* pair-rule network. For example, as diagramed in Fig. 8, we can imagine that after duplication of the ancestral *slp* gene, one copy continued to function as a segment polarity gene, but lost its pair-rule function, and did not diverge much at the sequence level (*Dm-slp2*). The other copy, while continuing to function as a pair-rule gene required for the activation of *wg*, is now required in even-numbered parasegments in *Drosophila*. In addition it has diverged at the sequence level (*Dm-slp1*). Furthermore, *opa* functions to activate *wg* in the odd-numbered parasegments in *Drosophila* while *ftz* is required to activate *en* in even-numbered parasegments (Benedyk et al., 1994; Ingham et al., 1988). Neither *opa* nor *ftz* has a pair-rule function in *Tribolium* (Choe et al., 2006), and in *Schistocerca ftz* is not even expressed segmentally (Dawes et al., 1994). Thus *ftz* and *opa* may have been co-opted as secondary pair-rule genes in the lineage leading to *Drosophila*. Alternatively, considering the fact that *Tc-ftz* is expressed in a pair-rule pattern in *Tribolium*, the possibility exists that its function in pair-rule patterning was lost in the beetle lineage. However, if the segment polarity function of *slp*, which is conserved in both insects, is considered to be the ancestral function, then it is possible that the pair-rule functions of *slp* in *Drosophila* and *Tribolium* are both derived. The two secondary pair-rule genes, *prd* and *slp* display conserved and divergent aspects in their regulation of segment polarity genes. The expression as well as the function of *prd* homologs in the formation of odd-numbered segments is conserved between *Drosophila* and *Tribolium*. In contrast, differences in the functional register of *slp* and the acquisition or loss of *ftz* and *opa* pair-rule functions are significant to the evolution of secondary pair-rule gene interactions. Functional analysis of homologs of *prd*, *slp*, *ftz*, and *opa* in other insects and basally branching arthropods are needed to test these models for the evolution of roles of secondary pair-rule genes in segmentation.

Acknowledgments

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