THE ROLE OF PAIR-RULE GENES IN *TRIBOLIUM* SEGMENTATION

by

CHONG PYO CHOE

B.S., Seoul National University, Seoul, Korea, 1997
M.S., Seoul National University, Seoul, Korea, 1999

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2006

Abstract

All arthropods share a segmented body plan. Detailed studies on segmentation mechanisms in the long-germ insect *Drosophila melanogaster* identified a segmentation hierarchy composed of maternal, gap, pair-rule, and segment polarity genes. In this hierarchy, pair-rule genes play an important role to translate gradients of regional information from maternal and gap genes into segmental expression of segment polarity genes. However, our understanding of the role of pair-rule genes in other short-germ insects and basally branching arthropods is still limited.

To gain insights into the role of pair-rule genes in short-germ segmentation, I analyzed genetic interactions as well as expression patterns and functions of homologs of *Drosophila* pair-rule genes in the short-germ insect *Tribolium castaneum*. Interestingly, despite the pair-rule like expression patterns of *Tribolium* homologs of almost all eight canonical *Drosophila* pair-rule genes, only five have a segmentation function. Knock-down of primary pair-rule genes caused asegmental and truncated phenotypes while knock-down of secondary pair-rule genes caused typical pair-rule phenotypes. Epistatic analysis between the genes revealed that primary pair-rule genes form a gene circuit to prepattern a two-segmental unit, and secondary pair-rule genes are downstream targets of the gene circuit.

The typical pair-rule phenotypes observed in secondary pair-rule gene RNAi embryos led to a detailed comparative analysis of the role of *paired* (*prd*) and *sloppy-paired* (*slp*) between *Drosophila* and *Tribolium*. This study revealed that *prd* is

functionally conserved while the functional parasegmental register for *Tribolium slp* is opposite that of *Drosophila slp*. 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.

Despite this flexibility in pair-rule patterning between *Drosophila* and *Tribolium*, segmental expression of *engrailed* (*en*) and *wingless* (*wg*) at parasegmental boundaries is conserved in both insects. Analysis of double and triple RNAi for pair-rule genes in *Tribolium* revealed that the primary pair-rule genes *even-skipped* and *runt* are redeployed to directly regulate *en* and *wg* with *prd* or *slp* at parasegmental boundaries. This redeployment of primary pair-rule genes seem to compensate for the apparently fewer number of functional secondary pair-rule genes in *Tribolium* segmentation.

THE ROLE OF PAIR-RULE GENES IN *TRIBOLIUM* SEGMENTATION

by

CHONG PYO CHOE

B.S., Seoul National University, Seoul, Korea, 1997
M.S., Seoul National University, Seoul, Korea, 1999

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2006

Approved by:

Major Professor
Dr. Susan Brown

Abstract

All arthropods share a segmented body plan. Detailed studies on segmentation mechanisms in the long-germ insect *Drosophila melanogaster* identified a segmentation hierarchy composed of maternal, gap, pair-rule, and segment polarity genes. In this hierarchy, pair-rule genes play an important role to translate gradients of regional information from maternal and gap genes into segmental expression of segment polarity genes. However, our understanding of the role of pair-rule genes in other short-germ insects and basally branching arthropods is still limited.

To gain insights into the role of pair-rule genes in short-germ segmentation, I analyzed genetic interactions as well as expression patterns and functions of homologs of *Drosophila* pair-rule genes in the short-germ insect *Tribolium castaneum*. Interestingly, despite the pair-rule like expression patterns of *Tribolium* homologs of almost all eight canonical *Drosophila* pair-rule genes, only five have a segmentation function. Knock-down of primary pair-rule genes caused asegmental and truncated phenotypes while knock-down of secondary pair-rule genes caused typical pair-rule phenotypes. Epistatic analysis between the genes revealed that primary pair-rule genes form a gene circuit to prepattern a two-segmental unit, and secondary pair-rule genes are downstream targets of the gene circuit.

The typical pair-rule phenotypes observed in secondary pair-rule gene RNAi embryos led to a detailed comparative analysis of the role of *paired* (*prd*) and *sloppy-paired* (*slp*) between *Drosophila* and *Tribolium*. This study revealed that *prd* is

functionally conserved while the functional parasegmental register for *Tribolium slp* is opposite that of *Drosophila slp*. 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.

Despite this flexibility in pair-rule patterning between *Drosophila* and *Tribolium*, segmental expression of *engrailed* (*en*) and *wingless* (*wg*) at parasegmental boundaries is conserved in both insects. Analysis of double and triple RNAi for pair-rule genes in *Tribolium* revealed that the primary pair-rule genes *even-skipped* and *runt* are redeployed to directly regulate *en* and *wg* with *prd* or *slp* at parasegmental boundaries. This redeployment of primary pair-rule genes seem to compensate for the apparently fewer number of functional secondary pair-rule genes in *Tribolium* segmentation.

Table of Contents

List of Figures

List of Table

Acknowledgements

I would like to thank my major advisor, Dr. Susan Brown for her guidance and endless support. I also wish to thank the other members of my committee, Dr. Denell, Dr. Herman, Dr. Clem, Dr. Zolkiewski, and Dr. Bowden for their valuable encouragements. I thank the members of the Brown and Denell labs for valuable discussions and critical reading of the manuscripts. I am grateful to Kathy Leonard in the insect room for providing beetles. Most of all, I do thank my parents and my sister for their endless love and support.

Introduction

All arthropods share a segmented body plan. Most of our current knowledge about segmentation mechanisms comes from works in the fruit fly *Drosophila melanogaster*. In *Drosophila*, a hierarchical cascade leads to progressive subdivision of embryos to generate reiterated segments (Lawrence, 1992). Initially, maternal coordinate genes establish embryonic polarity along the anterior-posterior axis (St Johnston and Nusslein-Volhard, 1992), and then concentration gradients of these genes differentially regulate expression of gap genes in different regions of embryos (Lawrence, 1992). Gap genes subdivide embryos into regions spanning several segments (Hulskamp and Tautz, 1991). Combinations of maternal coordinate genes and gap genes regulate expression of pair-rule genes in a double segment periodicity (Fujioka et al., 1999). Pair-rule genes are classified into two groups; primary pair-rule genes that are directly regulated by combinations of maternal coordinate genes and gap genes, and secondary pair-rule genes that are generally regulated by primary pair-rule genes (Peel et al., 2005). Pair-rule genes regulate expression of segment polarity genes in single segment periodicity to form each segment (Ingham et al., 1988). Through this segmentation hierarchy, fly segments are determined almost simultaneously in precellular blastoderm stages, which is a feature of long-germ mode of embryogenesis (St Johnston and Nusslein-Volhard, 1992).

Mutants of most segmentation genes displayed larval cuticular phenotypes that can be readily explained by the expression patterns of the genes in embryos. Mutant phenotypes of the anterior determinant *bicoid* (*bcd*) showed that head and thorax

development are defective or are replaced with by duplicated telson structures (Mohler and Wieschaus, 1985). The most severe mutant phenotypes of the posterior determinant *caudal* displayed the missing of most abdominal segments (Macdonald and Struhl, 1986). Mutations in gap genes lead to phenotypes in which several contiguous segments are missing or defective where gap genes are normally expressed (Peel et al., 2005). Interestingly, in most pair-rule genes mutant phenotypes, every other segment is missing or segmental defects occur in patterns of double segment periodicity, which is consistent with the double segmental expression patterns of pair-rule genes (Coulter and Wieschaus, 1988). In segment polarity gene mutants, a portion of each segment is defective or missing while the polarity of the remaining portions is reversed (Martizez Arias et al., 1988). Thus, generally speaking, mutant phenotypes of most segmentation genes are consistent with their expression patterns in fly embryos.

However, from an evolutionary point of view, the long-germ mode of embryogenesis of the fly is derived rather than ancestral (Davis and Patel, 2002). In contrast, embryogenesis of most insects and basally branching arthropods follows a short-germ mode in which only few anterior segments are predetermined at precellular blastoderm stages. The remaining segments form sequentially from a so called “growth zone” in a cellular environment during secondary germband growth phase (Davis and Patel, 2002). The fundamental morphological differences between long- and short-germ modes of segmentation have raise many questions concerning the genetic and molecular mechanisms of short-germ segmentation.

Analysis of the expression patterns of homologs of *Drosophila* segmentation genes in various insects during the last decade strongly suggested that the

segmentation hierarchy identified in the fly is largely conserved in other insects (Peel et al., 2005). However, lack of the anterior determinant *bcd* outside of dipteran insects suggests that the genetic and molecular mechanisms of segmentation in other insects and arthropods may not necessarily be the same as those of *Drosophila* (Stauber et al., 1999). Indeed, recent functional analysis of homologs of *Drosophila* segmentation genes in nondrosophilids using RNA interference (RNAi) reveals noncanonical as well as canonical functions in segmentation. For example, in the red flour beetle *Tribolium castaneum* and the wasp *Nasonia vitripennis*, *orthodenticle* and *hunchback* function as anterior determinants and may reflect an ancestral patterning mechanism replaced by *bcd* in *Drosophila* (Lynch et al., 2006; Schroder, 2003). Furthermore, RNAi for gap genes in *Tribolium*, the milkweed bug *Oncopeltus fasciatus*, and the cricket *Gryllus bimaculatus* caused homeotic transformation by misregulating Hox genes, as well as segmentation defects (Cerny et al., 2005; Liu and Kaufman, 2004; Mito et al., 2005). In addition, the *Oncopeltus* homolog of the *Drosophila* pair-rule gene *even-skipped* (*eve*) is expressed segmentally rather than pair-rule-like, and RNAi of *Of-eve* caused an asegmental and truncated phenotype rather than a typical pair-rule phenotype (Liu and Kaufman, 2005). However, despite variations in the roles of segmentation genes at levels upstream of segment polarity genes in the segmentation hierarchy, the segmental expression of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) at parasegmental boundaries is conserved in all arthropods examined thus far (Peel et al., 2005).

Although many aspects of the roles of segmentation genes in other insects still remain to be answered, the role of pair-rule genes in short-germ segmentation in

particular has been a long-standing question (Davis and Patel, 2003). In the long-germ *Drosophila*, the pair-rule genes play an essential role in the progressive subdivision of embryos during the simultaneous formation of segment. However, the need for pair-rule genes to define a two-segmental unit before the formation of individual segments during short-germ segmentation was not clear because the segments appear sequentially. *Tribolium castaneum* has played an important role during last decade as a model system to understand the role of pair-rule genes in short-germ segmentation. The discovery of pair-rule like expression patterns of the *Tribolium* homologs of *Drosophila hairy*, *eve* and *fushi tarazu* (*ftz*) has been considered strong evidence supporting the hypothesis that this short-germ insect would have similar pair-rule patterning to that of *Drosophila* (Brown et al., 1994; Brown et al., 1997; Patel et al., 1994; Sommer and Tautz, 1993). Furthermore, successful isolation of typical pair-rule mutants, like *scratchy* and *itchy*, in EMS-based genetic screens provided additional support for this hypothesis (Maderspacher et al., 1998). However, despite a key role of *ftz* in *Drosophila* pair-rule patterning as an activator of *en* stripe in even-numbered parasegments (Ingham et al., 1988), *Tc-ftz* is not involved in *Tribolium* segmentation (Stuart et al., 1991). Furthermore, RNAi for *Tc-eve* or *Tc-runt* caused almost asegmental and truncated phenotypes, which are dramatically different from the typical pair-rule phenotypes described in *Drosophila* (unpublished data in the Brown Lab). In summary, accumulating data on *Tribolium* pair-rule genes during the last decade suggest that there is pair-rule patterning in *Tribolium* segmentation but that the genetic and molecular mechanisms of pair-rule patterning would be different from those of *Drosophila*.

The goal of my dissertation research has been to understand the role of *Tribolium* homologs of all *Drosophila* pair-rule genes in *Tribolium* segmentation. For this purpose, I analyzed the genetic interactions between *Tribolium* pair-rule genes based on epistatic analysis, as well as the expression patterns and segmentation functions of individual pair-rule genes in *Tribolium*.

This dissertation is composed of three manuscripts. The first manuscript describes genetic interactions between *Tribolium* pair-rule genes to explain the RNAi phenotype of each pair-rule gene in *Tribolium* (Choe et al., 2006). The second manuscript focuses on detailed analysis of functions of *Tc-paired* and *Tc-sloppy-paired* as well as their expression patterns with potential molecular characterization of the pair-rule mutants *scratchy* and *itchy* (Choe and Brown, 2006). The third manuscript includes regulation of *Tc-en* and *Tc-wg* at parasegmental boundaries by *Tribolium* pair-rule genes (Choe and Brown, *submitted*).

References

Brown, S. J., Hilgenfeld, R. B. and Denell, R. E. (1994). The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc.Natl.Acad.Sci.U.S.A* **91**, 12922-12926.

Brown, S. J., Parrish, J. K., Beeman, R. W. and Denell, R. E. (1997). Molecular characterization and embryonic expression of the *even-skipped* ortholog of *Tribolium castaneum*. *Mech.Dev.* **61**, 165-173.

Cerny, A. C., Bucher, G., Schroder, R. and Klingler, M. (2005). Breakdown of abdominal patterning in the *Tribolium Kruppel* mutant *jaws*. *Development* **132**, 5353-5363.

Choe, C. P. and Brown, S. J. (2006). 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*. *Dev Biol* doi:10.1016/j.ydbio.2006.09.037

Choe, C. P., Miller, S. C. and Brown, S. J. (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. *Proc Natl Acad Sci U S A* **103**, 6560-4.

Coulter, D. E. and Wieschaus, E. (1988). Gene activities and segmental patterning in *Drosophila*: analysis of *odd-skipped* and pair-rule double mutants. *Genes Dev.* **2**, 1812-1823.

Davis, G. K. and Patel, N. H. (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annu.Rev.Entomol.* **47**, 669-699.

Davis, G. K. and Patel, N. H. (2003). Playing by pair-rules? *Bioessays* **25**, 425-9.

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B. (1999). Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* **126**, 2527-38.

Hulskamp, M. and Tautz, D. (1991). Gap genes and gradients--the logic behind the gaps. *Bioessays* **13**, 261-8.

Ingham, P. W., Baker, N. E. and Martinez-Arias, A. (1988). Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped*. *Nature* **331**, 73-75.

Lawrence, P. A. (1992). The Making of a Fly: the Genetics of Animal Design. Oxford, UK: Blackwell Scientific.

Liu, P. Z. and Kaufman, T. C. (2004). *hunchback* is required for suppression of abdominal identity, and for proper germband growth and segmentation in the intermediate germband insect *Oncopeltus fasciatus*. *Development* **131**, 1515-27.

Liu, P. Z. and Kaufman, T. C. (2005). *even-skipped* is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect. *Development* **132**, 2081-2092.

Lynch, J. A., Brent, A. E., Leaf, D. S., Pultz, M. A. and Desplan, C. (2006). Localized maternal *orthodenticle* patterns anterior and posterior in the long germ wasp *Nasonia*. *Nature* **439**, 728-32.

Macdonald, P. M. and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**, 537-45.

Maderspacher, F., Bucher, G. and Klingler, M. (1998). Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. *Dev.Genes Evol.* **208**, 558-568.

Martizez Arias, A., Baker, N. E. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 157-70.

Mito, T., Sarashina, I., Zhang, H., Iwahashi, A., Okamoto, H., Miyawaki, K., Shinmyo, Y., Ohuchi, H. and Noji, S. (2005). Non-canonical functions of *hunchback* in segment patterning of the intermediate germ cricket *Gryllus bimaculatus*. *Development* **132**, 2069-2079.

Mohler, J. and Wieschaus, E. F. (1985). *Bicaudal* mutations of *Drosophila melanogaster*: alteration of blastoderm cell fate. *Cold Spring Harb Symp Quant Biol* **50**, 105-11.

Patel, N. H., Condron, B. G. and Zinn, K. (1994). Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. *Nature* **367**, 429-434.

Peel, A. D., Chipman, A. D. and Akam, M. (2005). Arthropod segmentation: beyond the *Drosophila* paradigm. *Nat Rev Genet* **6**, 905-16.

Schroder, R. (2003). The genes *orthodenticle* and *hunchback* substitute for *bicoid* in the beetle *Tribolium*. *Nature* **422**, 621-5.

Sommer, R. J. and Tautz, D. (1993). Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* **361**, 448-450.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-19.

Stauber, M., Jackle, H. and Schmidt-Ott, U. (1999). The anterior determinant *bicoid* of *Drosophila* is a derived Hox class 3 gene. *Proc Natl Acad Sci U S A* **96**, 3786-9.

Stuart, J. J., Brown, S. J., Beeman, R. W. and Denell, R. E. (1991). A deficiency of the homeotic complex of the beetle *Tribolium*. *Nature* **350**, 72-4.

CHAPTER 1 - A pair-rule gene circuit defines segments sequentially in the short-germ insect, *Tribolium castaneum*

Chong Pyo Choe, Sherry C. Miller and Susan J. Brown*

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

***Corresponding author**: Susan J. Brown

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

Email: sjbrown@ksu.edu, Phone: (785) 532-3935, Fax: (785) 532-6653

Abbreviations: Engrailed (En), *even-skipped* (*eve*), *runt* (*run*), *odd-skipped* (*odd*), *paired* (*prd*), *sloppy-paired* (*slp*), *hairy* (*h*), *fushi-tarazu* (*ftz*)

Data deposition: *Tribolium castaneum* pair-rule gene sequences have been deposited in GenBank under accession numbers DQ414246 (*Tc-odd*), DQ414247 (*Tc-prd*), DQ414248 (*Tc-slp*).

Abstract

In *Drosophila*, a hierarchy of maternal, gap, pair-rule and segment polarity gene interactions regulates virtually simultaneous blastoderm segmentation. For the last decade, studies have focused on revealing the extent to which *Drosophila* segmentation mechanisms are conserved in other arthropods where segments are added sequentially from anterior to posterior in a cellular environment. Despite our increased knowledge of individual segmentation genes, details of their interactions in non-Drosophilid insects are not well understood. We analyzed the *Tribolium* orthologs of *Drosophila* pair-rule genes, which display pair-rule expression patterns. *Tribolium paired* (*Tc-prd*) and *sloppy-paired* (*Tc-slp*) produced pair-rule phenotypes when their transcripts were severely reduced by RNAi. In contrast, similar analysis of *Tribolium even-skipped* (*Tc-eve*), *runt* (*Tc-run*), or *odd-skipped* (*Tc-odd*) produced severely truncated, almost completely asegmental phenotypes. Analysis of interactions between pair-rule components revealed that *Tc-eve*, *Tc-run* and *Tc-odd* form a three-gene circuit to regulate one another as well as their downstream targets, *Tc-prd* and *Tc-slp*. The complement of primary pair-rule genes in *Tribolium* differs from *Drosophila* in that it includes *Tc-odd*, but not *Tc-hairy*. This gene circuit defines segments sequentially, in double segment periodicity. Furthermore, this single mechanism functions in the early blastoderm stage and subsequently during germband elongation. The periodicity of the *Tribolium* pair-rule gene interactions reveals components of the genetic hierarchy that are regulated in a repetitive circuit or clock-like mechanism. This pair-rule gene circuit

provides new insight into short-germ segmentation in *Tribolium* that may be more generally applicable to segmentation in other arthropods.

Introduction

In *Drosophila*, a hierarchy of maternal, gap, pair-rule and segment polarity genes regulates segmentation (1). Pair-rule genes transform regional gradients of maternal and gap gene information into cellular domains that define parasegmental boundaries (2), ultimately producing segments via regulation of segment-polarity genes. Genetic and molecular analyses reveal a complex pair-rule gene network, which operates in units of double segment periodicity. *even-skipped* (*eve*), *hairy* (*h*) and *runt* (*run*) are essential in setting parasegmental boundaries. These primary pair-rule genes are regulated by the maternal and gap genes, while they in turn regulate other, secondary pair-rule genes such as *fushi-tarazu* (*ftz*), *paired* (*prd*), *sloppy-paired* (*slp*) and *odd-skipped* (*odd*) (3, 4). In general, loss of primary pair-rule gene function affects the expression of secondary pair-rule genes, while the expression of primary pair-rule genes is not altered in secondary pair-rule gene mutants.

Comparative studies of pair-rule gene homologs in other insects reveal a wide variety of expression patterns. In the grasshopper *Schistocerca*, homologs of *eve* and *ftz* are not expressed in pair-rule stripes (5, 6). In the milkweed bug *Oncopeltus fasciatus*, the *eve* homolog is expressed in segmental, not pair-rule stripes (7). In the beetle *Tribolium castaneum*, where *eve*, *ftz*, *h* and *run* orthologs are expressed in pair-rule stripes (8-10), loss of *ftz* does not produce a pair-rule phenotype (11). However, pair-rule expression of *prd* homologs is conserved in *Drosophila*, *Tribolium* and *Schistocerca* (12). These results suggest that if insect segments are prepatterned in

units of double segment periodicity, then the genetic regulatory interactions of pair-rule mechanisms differ in each species.

Interactions among pair-rule genes in insects other than *Drosophila* have not been investigated to date. We have used parental RNA interference (RNAi) (13) to functionally analyze pair-rule gene orthologs and their interactions in the short-germ beetle *Tribolium castaneum*. Here we describe the genetic interactions of pair-rule patterning in the short-germ insect *Tribolium castaneum* and discuss implications for insect segmentation.

Results

Two classes of pair-rule genes in *Tribolium*.

Classic pair-rule mutant phenotypes in *Drosophila* include loss of alternating segments or defects displaying double segment periodicity, which are consistent with the normal expression pattern of the corresponding gene. Since *Tribolium* orthologs of these genes are expressed in pair-rule patterns, (see Fig. 1.5 and Supporting Results for expression of *Tc-odd*, *Tc-prd* and *Tc-slp*, which are published as supporting information on the PNAS web site) we expected RNAi to produce similar phenotypes. Surprisingly, however, strong knock-down of *Tc-eve*, *Tc-run* or *Tc-odd* transcripts produced truncated, almost completely asegmental embryos instead of pair-rule phenotypes. *Tc-eve*^{RNAi} embryonic cuticles contain labrum, antennae and telson (Fig. 1.1*b*), but no gnathal or trunk segments. In addition to labrum and antennae, *Tc-run*^{RNAi} cuticles contain mandibles (Fig. 1.1*c*), while *Tc-odd*^{RNAi} cuticles contain mandibles and maxilla (Fig.

1.1*d*). Consistent with these phenotypes, there are no gnathal or trunk Tc-En stripes in *Tc-eve*^{RNAi} germband embryos, and only one (mandibular) or two gnathal (mandibular and maxillary) stripes in *Tc-run* and *Tc-odd* RNAi embryos (Fig. 1.2 *g*, *m* and *s*) respectively. The homeotic gene *Tc-Dfd*, which serves as a molecular marker for mandibular and maxillary segments, is expressed normally in *Tc-odd*^{RNAi} embryos (Fig. 1.2*s*). Knock-down of any of these three genes blocked segmentation and elongation. In *Drosophila*, *eve* null mutants produce asegmental cuticles while null mutants of *run* or *odd* cause typical pair-rule phenotypes (14). The similar truncated, asegmental phenotypes of *Tc-eve*^{RNAi}, *Tc-run*^{RNAi} and *Tc-odd*^{RNAi} embryos suggest that these genes function at the same level in the segmentation hierarchy.

In contrast, *Tc-prd*^{RNAi} and *Tc-slp*^{RNAi} generated typical pair-rule phenotypes (Fig. 1.1 *e* and *f*) that phenocopy previously described mutants (15). Similar to *Drosophila prd* mutants (14), *Tc-prd*^{RNAi} embryonic cuticles lacked odd-numbered segments including mandibular, labial, T2 and four abdominal segments (Fig. 1.1*e*). Corresponding germband embryos lacked odd-numbered Tc-Engrailed (En) stripes (Fig. 1.3 *c* and *d*) suggesting that *Tc-prd* is essential for the expression of Tc-En in odd-numbered parasegments. Complementary to *Tc-prd*^{RNAi}, *Tc-slp*^{RNAi} cuticles lacked even-numbered segments (Fig. 1.1*f*). Corresponding germband embryos lacked even-numbered Tc-En stripes (Fig. 1.3 *e* and *f*) indicating that *Tc-slp* is required for the expression of Tc-En in even-numbered parasegments. Interestingly, hypomorphic *slp* mutants in *Drosophila* affects of odd-numbered segments (16), whereas *Tc-slp*^{RNAi} affects even-numbered segments, implying that the requirement for *slp* function is different in flies and beetles.

The two classes of cuticular phenotypes seen in RNAi embryos suggest that in *Tribolium*, pair-rule genes may operate at two functional levels, as in *Drosophila*. In addition, nascent stripes of *Tc-run* and *Tc-odd* appear in the posterior growth zone, while stripes of *Tc-prd* and *Tc-slp* appear later in the anterior growth zone (see Fig. 1.4*a* and Supporting Results, which are published as supporting information on the PNAS web site). Taken together, these data suggest that *Tc-eve*, *Tc-run* and *Tc-odd* may function as primary pair-rule genes while *Tc-prd* and *Tc-slp* function as secondary pair-rule genes.

We also analyzed the functions of the remaining candidate pair-rule genes, *Tribolium h*, *ftz*, *odd-paired* (*opa*) and *Tenascin major* (*Ten-m*). However, no segmentation defects were observed (data not shown), with the exception of *Tc-h*^{RNAi}, which produced anterior defects (Fig. 1.6 and Supporting Results, which are published as supporting information on the PNAS web site). The truncated, asegmental phenotypes shown by *Tc-eve*^{RNAi}, *Tc-run*^{RNAi} and *Tc-odd*^{RNAi} embryos, the modified pair-rule function of *Tc-slp* and the fact that not all pair-rule gene orthologs participate in segmentation in *Tribolium* strongly suggest that segments are prepatterned by different pair-rule genes interactions in *Tribolium* and *Drosophila*.

Epistasis analysis of *Tribolium eve*, *run* and *odd*.

To understand how genes expressed in pair-rule stripes produce truncated and asegmental RNAi embryonic cuticles, we examined the RNAi effects of each gene on the expression of the others. In strong *Tc-eve*^{RNAi} embryos, expression of *Tc-run* and *Tc-odd* was lost or greatly reduced, indicating *Tc-eve* is required for the activation of *Tc-*

run and *Tc-odd* (Fig. 1.2 *h-j*). The expression patterns of *Tc-eve* and *Tc-odd* are almost completely complementary and show only slight overlap (Fig. 1.5*b*). Therefore, *Tc-eve* probably indirectly activates *Tc-odd*. In severe *Tc-odd*^{RNAi} embryos, the broad initial expression domains of *Tc-eve* and *Tc-run* failed to resolve into pair-rule stripes (Fig. 1.2 *t-v*). Thus *Tc-odd* is required for repression of *Tc-eve* and *Tc-run* to produce pair-rule stripes. However, it is unlikely that *Tc-odd* directly represses *Tc-run* since their expression patterns overlap (Fig. 1.4a and Supporting Results). Instead, *Tc-odd* might repress *Tc-run* through repression of *Tc-eve*. In *Drosophila*, the initial expression of the primary pair-rule genes *eve* and *run*, is not altered by mutations in *odd* (17), a secondary pair-rule gene. The ectopic expression of *Tc-eve* and *Tc-run* in *Tc-odd*^{RNAi} indicates that different genetic interactions between these genes evolved in the lineages leading to beetles and flies. Strong *Tc-run*^{RNAi} caused broad expression of *Tc-eve* as well as severe reduction of *Tc-odd* expression in the growth zone implying that *Tc-run* is required for activation of *Tc-odd* and repression of *Tc-eve* (Fig. 1.2 *n-p*). However, the overlap between *Tc-eve* and *Tc-run* expression (Fig. 1.7, which is published as supporting information on the PNAS web site) suggests that the repression of *Tc-eve* by *Tc-run* is an indirect effect mediated by *Tc-odd*. These interactions indicate that these three genes provide primary pair-rule functions in *Tribolium*.

Tribolium prd and *slp* are secondary pair-rule genes.

To understand whether *Tc-prd* and *Tc-slp* function as primary or secondary pair-rule genes, we analyzed the effect of *Tc-prd* or *Tc-slp* RNAi on the expression of the others. The expression of *Tc-eve*, *Tc-run* or *Tc-odd* was not altered in *Tc-prd*^{RNAi} or *Tc-slp*^{RNAi}

embryos (data not shown). However, the stripes of *Tc-prd* and *Tc-slp* failed to resolve in *Tc-eve*^{RNAi} and *Tc-run*^{RNAi} embryos (Fig. 1.2 *k*, *l*, *q* and *r*), probably due to the absence of inter-stripe repression. In contrast, *Tc-prd* and *Tc-slp* expression was abolished in the growth zone of *Tc-odd*^{RNAi} embryos (Fig. 1.2 *w* and *x*), suggesting that *Tc-prd* and *Tc-slp* provide pair-rule functions that are secondary to those of *Tc-eve*, *Tc-run* and *Tc-odd*. In addition, *Tc-prd* was expressed normally in *Tc-slp*^{RNAi} and *Tc-slp* was expressed normally in *Tc-prd*^{RNAi} embryos (data not shown), indicating that they do not interact with each other and are in parallel positions in the pathway. Although *Tc-prd* and *Tc-slp* were misregulated by the knock-down of the three primary pair-rule genes, it seems likely that *Tc-eve* and *Tc-odd* regulate *Tc-prd* and *Tc-slp* indirectly through *Tc-run*; *Tc-prd* and *Tc-slp* were still expressed broadly in *Tc-run*^{RNAi} embryos (Fig. 1.2 *q* and *r*) in which *Tc-eve* was expressed ectopically and *Tc-odd* expression was abolished. These results place them downstream of *Tc-run*.

Tribolium pair-rule genes do not act upstream of gap genes.

Depletion of *eve* mRNA in the milkweed bug *Oncopeltus fasciatus* results in misregulation of gap genes, producing a severe head-only phenotype (7). To determine whether misregulation of gap genes contributed to the asegmental phenotypes observed in *Tc-eve*^{RNAi}, *Tc-run*^{RNAi}, and *Tc-odd*^{RNAi} embryos, we examined their expression in RNAi germband embryos. Expression of the *Tribolium* orthologs of *hunchback*, *Krüppel*, *giant* and *knirps*, are largely normal in the RNAi embryos (data not shown), suggesting that the asegmental phenotypes generated by RNAi for *Tribolium* pair-rule genes, are not due to the misregulation of *Tribolium* gap genes.

Discussion

We analyzed the functions and interactions of the *Tribolium* homologs of *Drosophila* pair-rule genes using RNAi. We discovered that the *Tribolium* homologs of *eve*, *run* and *odd* function as primary pair-rule genes and *prd* and *slp* function as secondary pair-rule genes but *h*, *ftz* and *opa* and *Ten-m* do not function as pair-rule genes. Severe knock-down of *Tribolium* primary pair-rule genes led to truncated, asegmental phenotypes, while depletion of secondary pair-rule genes produced classic pair-rule phenotypes. Based on these discoveries, we propose a model of pair-rule patterning in *Tribolium* that might explain the RNAi phenotypes and discuss major differences between in the interactions of pair-rule genes in *Drosophila* and *Tribolium*. Finally, we discuss the implications of these findings on segmentation in short germ insects and other arthropods.

A model of pair-rule gene interaction in *Tribolium*

We describe a pair-rule gene circuit in Fig. 1.4*a*, in which *Tc-eve* expression is required to activate *Tc-run*, which in turn is required to activate *Tc-odd*. *Tc-odd* expression in even-numbered parasegments is required to repress *Tc-eve* there, separating a primary *Tc-eve* stripe from the broad expression domain. As *Tc-eve* expression is repressed in even-numbered parasegments, the posterior edges of *Tc-run* and then *Tc-odd* expression fade. *Tc-eve* expression is also repressed in odd-numbered parasegments (regulated by an as yet unknown gene) to produce segmental *Tc-eve* secondary stripes that are coincident with En stripes (8, 18). Loss of *Tc-eve* expression in odd-numbered parasegments causes *Tc-run* stripes to fade from their anterior edge, resulting in narrow

Tc-run stripes that are coincident with every even-numbered En stripe. For reasons yet unknown, all three genes remain coexpressed with Tc-En in even-numbered parasegments. Consequently, a two-segment unit is prepatterned through one cycle of this primary pair-rule gene circuit. Restriction of *Tc-run* expression leads to the derepression of *Tc-prd* and *Tc-slp*, which are responsible for the activation of Tc-En in odd- and even-numbered parasegments, respectively.

The asegmental phenotypes produced by RNAi analysis of *Tc-eve*, *Tc-run* and *Tc-odd* are readily explained by this model. The knock-down of *Tc-eve* abolishes *Tc-run* expression, which induces ectopic expression of both *Tc-prd* and *Tc-slp*. Tc-En expression is not properly regulated to define the parasegmental borders, which results in an asegmental phenotype. Similarly for *Tc-run*^{RNAi}, in the absence of *Tc-run*, *Tc-prd* and *Tc-slp* are expressed ectopically, Tc-En is not activated and segmental grooves are not formed. However, the mechanism that generates the asegmental phenotype in *Tc-odd*^{RNAi} embryos is different from that in *Tc-eve*^{RNAi} or *Tc-run*^{RNAi} embryos; the knock-down of *Tc-odd* leads to ectopic expression of *Tc-eve*, which induces ectopic expression of *Tc-run*. As a result, *Tc-prd* and *Tc-slp* are fully repressed, which leads to misregulation of Tc-En expression and produces the asegmental *Tc-odd*^{RNAi} phenotype. Thus, either loss or ectopic expression of *Tc-prd* or *Tc-slp* leads to misregulation of Tc-En, ultimately resulting in asegmental phenotypes.

Major differences of pair-rule interactions between *Drosophila* and *Tribolium*

Our model of pair-rule interactions in *Tribolium* is not predicted by simple application of the *Drosophila* pair-rule gene paradigm (19) (Fig. 1.4*b*). In *Drosophila*, the three primary

pair-rule genes – *h*, *eve* and *run* – are key players to initiate pair-rule patterning. However, *Tc-h* seems not to function as a pair-rule gene at all. Although *odd* is a secondary pair-rule gene in *Drosophila* that is repressed by *eve*, *Tc-odd* functions as a primary pair-rule gene in *Tribolium* that represses *Tc-eve*. Repression of *slp* and *odd* by *eve* is critical to activate *prd*-dependent odd- and *ftz*-dependent even-numbered *en* stripes respectively in *Drosophila* (19, 20) (Fig. 1.4*b*). In contrast, *Tc-eve* is required for the activation of *Tc-odd*, which in turn represses *Tc-eve* to prepattern a two-segment unit. Furthermore, *Tc-run* which is induced by *Tc-eve*, is important for the formation of *Tc-prd*-dependent odd- and *Tc-slp*-dependent even-numbered *Tc-en* stripes. *Drosophila ftz* is a secondary pair-rule gene that activates even-numbered *en* stripes, but *Tc-ftz* does not function in segmentation (11). Differences in the primary pair-rule genes result in different genetic interactions between primary and secondary genes and likely affect the regulatory interactions between pair-rule and segment polarity genes. For example, loss of *slp* affects odd-numbered parasegments, while loss of *Tc-slp* affects even-numbered parasegments.

Our model provides a core mechanism for pair-rule patterning in *Tribolium* segmentation. However, additional components remain to be discovered. *Tc-eve*, *Tc-run* and *Tc-odd* have different anterior boundaries of expression that correspond to the number of gnathal segments remaining in RNAi embryos. These boundaries are likely regulated by gap genes, as in *Drosophila*.

Using the candidate gene approach we determined that orthologs of genes previously identified as pair-rule genes in *Drosophila* function in *Tribolium* segmentation. However, the gene(s) responsible for resolution of primary, pair-rule *Tc-eve* stripes into

secondary, segmental stripes as well as genes that limit the expression of *Tc-run* within the *Tc-eve* domain and *Tc-odd* within the *Tc-run* domain have yet to be determined. Furthermore, we do not yet know which genes function to activate *Tc-prd* and *Tc-slp*. Future studies must also determine how the pair-rule gene circuit is initiated in blastoderm embryos and stopped after elongation. If this pair-rule gene circuit is regulated by genes involved in anterior-posterior patterning, *Tc-caudal* is a likely candidate. It is strongly expressed in the growth zone throughout germband elongation (21, 22) and produces a severe RNAi phenotype (23) that is identical that described for *Tc-eve*. Gap genes such as *Tc-hunchback*, which is expressed in the posterior-most regions of the elongating germband (24), may be involved in regulating the pair-rule gene circuit there. On the other hand, since pair-rule patterning occurs in a cellular environment in *Tribolium*, it is possible that intercellular signaling pathways are involved in regulating the pair-rule gene circuit as components or targets of a segmentation clock. Indeed the sequential function of the pair-rule gene circuit during *Tribolium* segmentation is the first evidence for regulation by some type of periodic mechanism in insects. In vertebrates, somitogenesis is regulated by a segmentation clock (25). Homologs of vertebrate segmentation clock components, such as *Notch* and *Delta*, are required for proper segmentation in basal arthropods such as the spider *Cupiennius*, and have led to the speculation that this mode of segmentation might be very ancient (26). Although a *Notch* homolog has not been implicated in insect segmentation (27), other signaling molecules may provide the regulatory link between pair-rule genes and a segmentation clock.

Primary pair-rule genes in germband elongation

In *Tribolium*, a short, wide germ rudiment elongates into a long narrow germband during segmentation (28). In the absence of concerted cell division, this morphological change may be due to cell movement and intercalation similar to convergent extension in *Drosophila* (29). Germband elongation is not disrupted in *Tc-prd* and *Tc-slp* RNAi embryos; the classic pair-rule phenotypes result from loss of patterning in alternating segments. In contrast, defective elongation in *Tc-eve*, *Tc-run* and *Tc-odd* RNAi embryos produces short, amorphous germbands in which posterior segments are not initiated. These results, taken together with their wild type expression patterns, implicate primary (but not secondary) pair-rule genes in elongation as well as segmentation. Interestingly, *eve* and *run* have been implicated in convergent extension of the *Drosophila* germband (29).

One segmentation mechanism functions in the blastoderm and during elongation.

In *Tribolium*, up to three pair-rule stripes form in the cellular blastoderm, prepatterning the three gnathal and three thorax segments; abdominal segments are subsequently added from the growth zone during germband elongation. Gap gene RNAi and mutant phenotypes display specific homeotic phenotypes in the gnathum and thorax, while severely disrupting segmentation in the abdomen (30, 31). These results have led to the hypothesis that segmentation mechanisms differ between blastoderm and elongation phases of short-germ development. The pair-rule gene-circuit we describe prepatterns segments in double segment periodicity from the gnathum through the abdomen providing continuity between the blastoderm and germband elongation phases. Thus, it

appears that biggest difference between these phases occurs at the level of the gap genes.

Several insights into segmentation in other short-germ arthropods

Our results provide several insights into segmentation in *Tribolium* that may apply to other short-germ arthropods in general. First, a smaller complement of genes may comprise the core pair-rule mechanism. Second, primary and secondary genes may be different than in *Drosophila*. Indeed, the dynamics of pair-rule gene homolog expression in the spider, *Cupiennius* (32) suggest pair-rule gene functions that differ from those of their *Drosophila* counterparts. Third, if primary pair-rule genes function in both elongation and segmentation in short-germ arthropods, they may produce dramatically more severe RNAi phenotypes than secondary pair-rule genes. RNAi analysis in more non-model arthropods is required to test these insights and provide a better understanding of the logic of the ancestral pair-rule patterning mechanism.

Materials and Methods

Molecular Analysis

Tc-odd, *Tc-prd* and *Tc-slp* sequences were computationally identified in the *Tribolium* genome sequence by tBLASTn analysis of *Drosophila* protein sequences. PCR amplicons from total embryonic RNA were cloned to use as templates for in situ probes or dsRNA.

Parental RNAi

Parental RNAi was performed as described (13). Injection of 900 ng/µl (*Tc-eve*), 500 ng/µl (*Tc-run*, *Tc-prd* and *Tc-slp*) or 350 ng/µl (*Tc-odd*) into pupae produced strong RNAi effects. 1X injection buffer or 1µg/µl of *Tc-ftz* dsRNA were injected and produced no mutant effects.

Whole-mount in situ hybridization and immunocytochemistry

Whole-mount in situ hybridization was carried out as in (8, 9) with Digoxigenin-labeled RNA probes. The anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics) was preadsorbed and used at a 1/2000 dilution. Immunocytochemistry was performed as described in (8) with the anti-Eve diluted to 1/20 or the anti-En antibody diluted to 1/5. Germ-bands were dissected out from the yolks of embryos, were mounted in 80% glycerol and photographed by using Nomarski optics.

Phenotype analysis

Cuticle preparations of RNAi embryos were performed as described (11). First instar larvae were observed and photographed under dark-field optics.

Acknowledgements

We thank T.Shippy, Y.Tomoyasu, R.Denell and M.Klingler for valuable discussions and critical reading of the manuscript. We thank the Baylor Human Genome Sequencing Center for Tribolium genomic sequences (http://www.hgsc.bcc.tmc.edu). This work was supported by a grant from the National Institutes of Health (to S.J.B.).

Reference List

1. Ingham, P. W. (1988) *Nature* **335**, 25-34.
2. Niessing, D., Rivera-Pomar, R., La, R. A., Hader, T., Schock, F., Purnell, B. A. & Jackle, H. (1997) *J. Cell Physiol* **173**, 162-167.
3. Ingham, P. & Gergen, P. (1988) *Development* **104** Suppl., 51-60.
4. Martinez-Arias, A., Baker, N. & Ingham, P. (1988) *Development* **103**, 157-170.
5. Patel, N. H., Ball, E. E. & Goodman, C. S. (1992) *Nature* **357**, 339-342.
6. Dawes, R., Dawson, I., Falciani, F., Tear, G. & Akam, M. (1994) *Development* **120**, 1561-1572.
7. Liu, P. Z. & Kaufman, T. C. (2005) *Development* **132**, 2081-2092.
8. Patel, N. H., Condron, B. G. & Zinn, K. (1994) *Nature* **367**, 429-434.
9. Brown, S. J., Hilgenfeld, R. B. & Denell, R. E. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12922-12926.
10. Brown, S. J. & Denell, R. E. (1996) *Sem. Cell Dev. Biol.* **7**, 553-560.
11. Stuart, J. J., Brown, S. J., Beeman, R. W. & Denell, R. E. (1991) *Nature* **350**, 72-74.
12. Davis, G. K., Jaramillo, C. A. & Patel, N. H. (2001) *Development* **128**, 3445-3458.
13. Bucher, G., Scholten, J. & Klingler, M. (2002) *Curr. Biol.* **12**, R85-R86.
14. Coulter, D. E. & Wieschaus, E. (1988) *Genes Dev.* **2**, 1812-1823.
15. Maderspacher, F., Bucher, G. & Klingler, M. (1998) *Dev. Genes Evol.* **208**, 558-568.
16. Grossniklaus, U., Kurth Pearson, R. & Gehring, W. J. (1992) *Genes Dev.* **6**, 1030-1051.

17. Saulier-Le, D. B., Nasiadka, A., Dong, J. & Krause, H. M. (1998) *Development* **125**, 4851-4861.

18. Brown, S. J., Parrish, J. K., Beeman, R. W. & Denell, R. E. (1997) *Mech. Dev.* **61**, 165-173.

19. Jaynes, J. B. & Fujioka, M. (2004) *Dev. Biol.* **269**, 609-622.

20. Fujioka, M., Jaynes, J. B. & Goto, T. (1995) *Development* **121**, 4371-4382.

21. Wolff, C., Schroder, R., Schulz, C., Tautz, D. & Klingler, M. (1998) *Development* **125**, 3645-3654.

22. Schulz, C., Schröder, R., Hausdorf, B., Wolff, C. & Tautz, D. (1998) *Dev. Genes Evol.* **208**, 283-289.

23. Copf, T., Schroder, R. & Averof, M. (2004) *Proc. Natl. Acad. Sci. U. S. A* **101**, 17711-17715.

24. Wolff, C., Sommer, R., Schröder, R., Glaser, G. & Tautz, D. (1995) *Development* **121**, 4227-4236.

25. Pourquie, O. (2001) *Annu. Rev. Cell Dev. Biol.* **17**, 311-350.

26. Stollewerk, A., Schoppmeier, M. & Damen, W. G. (2003) *Nature* 423, 863-865.

27. Tautz, D. (2004) *Dev. Cell* **7**, 301-312.

28. Brown, S. J., Patel, N. H. & Denell, R. E. (1994) *Dev. Genet.* **15**, 7-18.

29. Irvine, K. D. & Wieschaus, E. (1994) *Development* **120**, 827-841.

30. Bucher, G. & Klingler, M. (2004) *Development* **131**, 1729-1740.

31. Sulston, I. A. & Anderson, K. V. (1996) *Development* **122**, 805-814.

32. Damen, W. G., Janssen, R. & Prpic, N. M. (2005) *Evol. & Dev.* **7**, 618-628.

Supporting Results

1. Expression patterns of *Tribolium* pair-rule genes

Tc-eve is initially expressed from 0-70% egg length as measured from the posterior (1). Primary stripes, which resolve within this domain by interstripe repression, initially span an odd-numbered parasegment and the adjacent even-numbered Tc-Engrailed (Tc-En) stripe (2). *Tc-run* is transiently expressed from 0-50% egg length in the blastoderm embryo. Each initially broad *Tc-run* stripe, centered over an even-numbered Tc-En stripe, resolves to exactly overlap that Tc-En stripe prior to fading away (3).

We identified *Tribolium* orthologs of each *Drosophila odd-skipped* (*odd*) family gene including *odd*, *sister of odd and bowl* (*sob*), *bowel* (*bowl*), and *drumstick* (*drm*). Only expression of *Tc-odd* was examined in this study. *Tc-odd* is initially expressed in even-numbered parasegments complementary to *Tc-eve*. Each *Tc-odd* stripe fades from posterior to anterior, and eventually fades away completely (Fig. 1.5 *a, b*). *Tc-eve* is continuously expressed throughout the posterior region of the embryo, including the growth zone. *Tc-run* and *Tc-odd* stripes appear de novo very near the posterior end of the embryo in the growth zone.

The mandibular *Tc-prd* stripe appears first. Subsequent *Tc-prd* stripes, whose graded expression is strongest posteriorly, initially span an even-numbered parasegment and the adjacent odd-numbered Tc-En stripe. The central region of *Tc-prd* primary stripes between two Tc-En stripes fades from anterior to posterior; producing one weak and one strong segmental *Tc-prd* stripe, coincident with even- and odd- Tc-En stripes, respectively (Fig. 1.5 *c*, *d*).

Two segmental *Tc-slp* stripes, one weak and one strong, appear virtually simultaneous. The stronger, posterior stripe is coincident with an even-number *Tc-wingless* (*Tc-wg*) stripe and the weaker, anterior stripe with an odd-numbered *Tc-wg* stripe (Fig. 1.5 *e*, *f*). *Tc-prd* and *Tc-slp* stripes appear de novo relatively distant from the posterior end of the embryo (in the anterior growth zone) prior to the appearance of Tc-En, and remain stably expressed throughout segmentation.

2. RNAi effects of *Tc-hairy*, *Tc-odd-paired* and *Tc-Tenascin major*

Severe *Tc-h*^{RNAi} embryos display anterior regions defects. In the most severe cases, the entire head and anterior thorax (through T2) were absent, but the remaining segments (T3 and all abdominal segments) were still normal. In addition, the expression pattern and function of the *Tribolium* homolog of *Drosophila deadpan* (*dpn*) were analyzed. *Tc-dpn* is not expressed in stripes during *Tribolium* segmentation. In addition, *Tc-dpn*^{RNAi} did not reveal any segmental defects.

Strong knock-down of *Tc-opa* or *Tc-Ten-m* transcripts caused high levels of embryonic lethality. Cuticles of the few embryos that did complete embryonic development were normal, and germbands displayed normal Tc-En expression. *Tc-ftz*^{RNAi} did not effect embryonic survival; cuticles and Tc-En stained germbands were normal.

3. Register of *Tribolium* pair-rule gene expression

The expression domains of the *Tribolium* pair-rule genes were determined relative to Tc-En in double-stained embryos. In addition, we determined the expression of *Tc-run*

and *Tc-odd* relative to Tc-Eve (Fig. 1.7 and Fig. 1.5*b*). These comparisons provide enough evidence to speculate that the primary *Tc-odd* stripes overlap the *Tc-run* stripes in the growth zone.

Reference List

1. Brown, S. J., Parrish, J. K., Beeman, R. W. & Denell, R. E. (1997) *Mech. Dev.* **61,** 165-173.
2. Patel, N. H., Condron, B. G. & Zinn, K. (1994) *Nature* **367,** 429-434.
3. Brown, S. J. & Denell, R. E. (1996) *Sem. Cell Dev. Biol.* **7,** 553-560.

Figures

Figure 1.1 Cuticle preparations of severe Tc-pair-rule gene RNAi embryos.

(*a*) This wild-type first instar larval cuticle contains head, three thoracic segments (T1–3), eight abdominal segments (A1-8) and terminal structures. Lr, labrum; Ant, antennae; Md, mandibles; Mx, maxillae; Lb, labium. (*b*) This spherical, asegmental *Tc-eve*^{RNAi} cuticle contains labrum and antennae, but no trunk segments. (*c*) In this severe *Tc-run*^{RNAi} cuticle, the preoral and mandibular segments developed normally, but all other segments are missing, resulting in a spherical body similar to that of the *Tc-eve*^{RNAi} embryo in *b*. (*d*) Preoral, mandibular and maxillary segments developed normally in this severe *Tc-odd*^{RNAi} cuticle, but the absence of posterior segments produced a spherical body shape similar to the *Tc-eve*^{RNAi} and *Tc-run*^{RNAi}. (*e*) This severe *Tc-prd*^{RNAi} cuticle contains maxillary, T1, T3 and four abdominal segments. (*f*) In this severe *Tc-slp*^{RNAi} cuticle, T2 and four abdominal segments formed, while all gnathal and even-numbered trunk segments are missing.

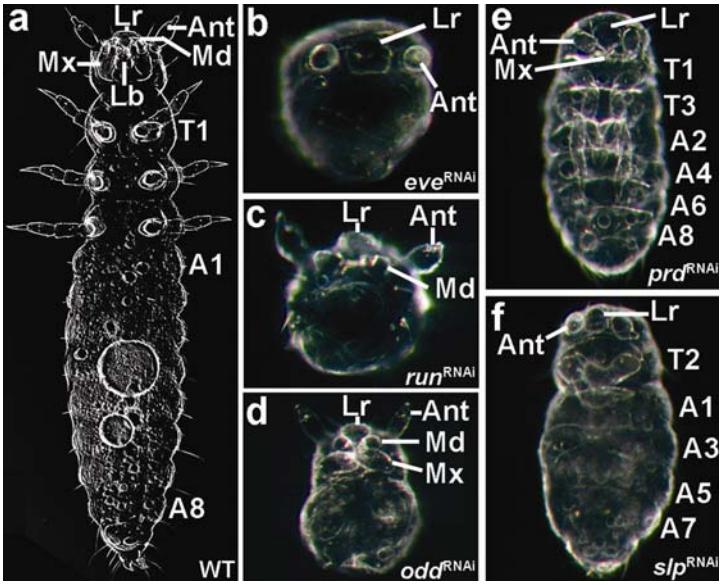


Figure 1.2 Expression of *Tribolium* pair-rule genes in primary pair-rule gene RNAi embryos.

(*a-f*) Expression of Tc-En and pair-rule genes in wild-type embryos; (*g-l*) in *Tc-eve*^{RNAi} embryos; (*m-r*) in *Tc-run*^{RNAi} embryos, and (*s-x*) in *Tc-odd*^{RNAi} embryos. (*g*) Antennal and intercalary Tc-En stripes formed in this severe *Tc-eve*^{RNAi} embryo. In severe *Tc-eve*^{RNAi} embryos, expression of *Tc-eve* (*h*), *Tc-run* (*i*) and *Tc-odd* (*j*) were severely reduced or abolished and *Tc-prd* (*k*) and *Tc-slp* (*l*) failed to resolve into stripes. (*m*) In this severe *Tc-run*^{RNAi} embryo, only antenna and mandibular Tc-En stripes formed. In severe *Tc-run*^{RNAi} embryos, *Tc-eve* (*n*), *Tc-prd* (*q*) and *Tc-slp* (*r*) were ectopically expressed but *Tc-run* (*o*) and *Tc-odd* (*p*) expression was strongly reduced. (*s*) *Tc-Deformed* (purple) and Tc-En are expressed normally in the mandibular and maxillary segments of this severe *Tc-odd*^{RNAi} embryo. In severe *Tc-odd*^{RNAi} embryos *Tc-eve* (*t*) and *Tc-run* (*u*) were expressed in broad continuous domains, but *Tc-odd* (*v*), *Tc-prd* (*w*) and *Tc-slp* (*x*) expression was abolished.

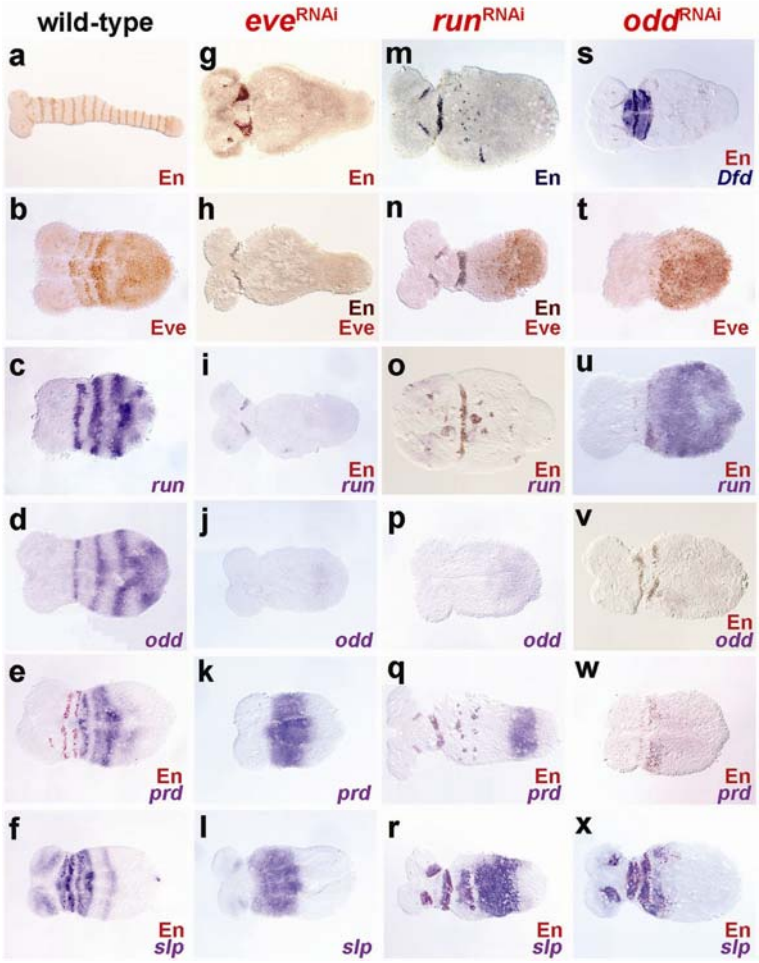


Figure 1.3 Tc-En staining reveals pair-rule defects in severe secondary Tc-pair-rule gene RNAi embryos.

(*a*) 16 Tc-En stripes are visible in this fully elongated wild-type germband. (*b*) *Tc-run* is transiently expressed in even-numbered parasegments in this elongating wt germband. (*c*) There are only 7 Tc-En stripes in this fully elongated *Tc-prd*^{RNAi} germband. (*d*) The Tc-En stripes overlap *Tc-run* stripes, indicating that the odd numbered Tc-En stripes are missing. (*e,f*) In this *Tc-slp*^{RNAi} embryo, all gnathal Tc-En stripes and every even-numbered Tc-En stripe in the trunk are missing.

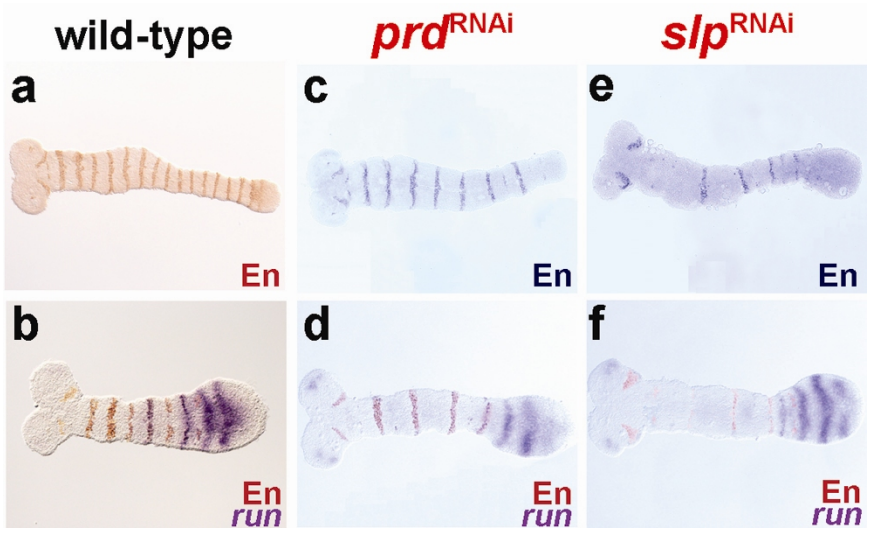
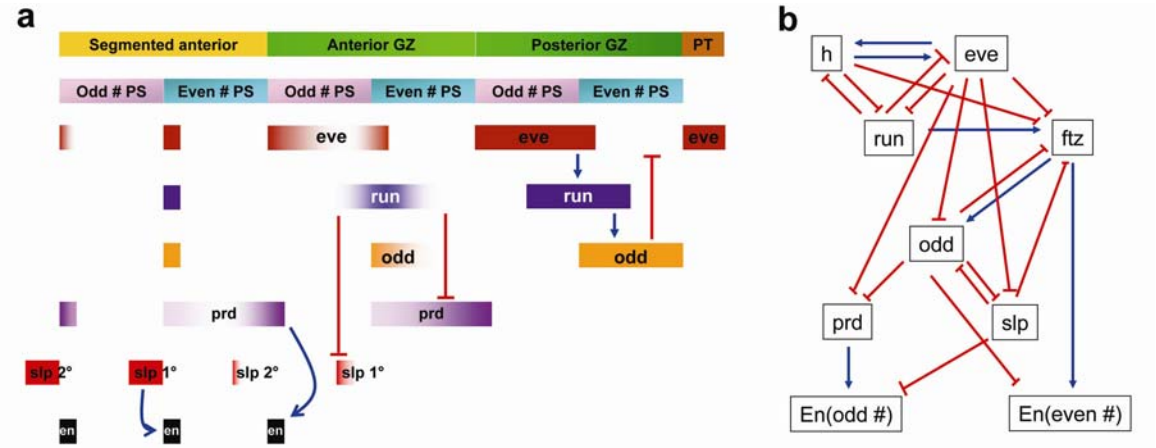


Figure 1.4 Pair-rule patterning in *Tribolium*.

(*a*) The dynamic expression of the primary and secondary pair-rule genes and their regulatory interactions are summarized in this figure. The bar at the top indicates anterior is to the left. Newer segments forming in the growth zone are to the right. In this model of pair-rule patterning in *Tribolium*, two-segment units are prepatterned in the posterior region of the growth zone through one cycle of the regulatory circuit (*Tc-eve*, *Tc-run*, *Tc-odd*). As the expression of *Tc-run* retracts anteriorly in even-numbered parasegments, the expression of *Tc-prd* is derepressed. Primary *Tc-prd* stripes resolve into two secondary stripes showing alternatively weak and strong segmental expression. The strong secondary stripes in odd-numbered parasegments regulate Tc-En expression. *Tc-run* also retracts posteriorly in odd-numbered parasegments resulting in derepression of the primary *Tc-slp* stripes. As *Tc-run* expression fades, expression of the primary *Tc-slp* stripe extends to the posterior border of odd-numbered parasegment, which is required for the initiation of Tc-En. GZ, growth zone; PS, parasegment; PT, posterior tip. (*b*) The more complex pair-rule network in *Drosophila* (19).



Supporting figures

Figure 1.5 Pair-rule expression of *Tc-odd*, *Tc-prd* and *Tc-slp* genes at early germ-band stages.

(*a*) Four stripes of *Tc-odd* mRNA expression in even-numbered parasegments are visible. The youngest, most posterior stripe initiates as two spots in the ectoderm flanking the central mesoderm. (*b*) Prior to fading, *Tc-odd* stripes are complementary to Tc-Eve stripes (Tc-Eve, gold; *Tc-odd*, purple). (*c*) Graded primary stripes of *Tc-prd* mRNA, spanning two Tc-En stripes, are stronger posteriorly (Tc-En, dark punctate spots within the *prd* expression domain; *Tc-prd*, purple). (*d*) Each primary *Tc-prd* stripe resolves into two secondary stripes that alternative in intensity. (*e* and *f*) A pair of segmental *Tc-slp* stripes appear together, one weak (arrow) and one strong (arrowhead), each overlaps the anterior boundary of a punctate Tc-En stripe in the anterior region of growth zone.

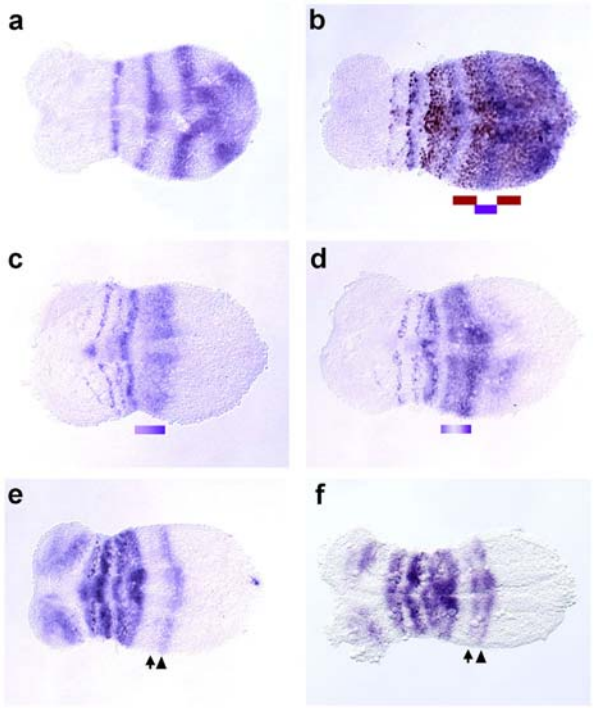


Figure 1.6 Severe *Tc-hairy*^{RNAi} cuticular phenotype.

Strong knock-down of the transcript for *Tc-h* revealed headless embryos with normal posterior thoracic and abdominal segments.

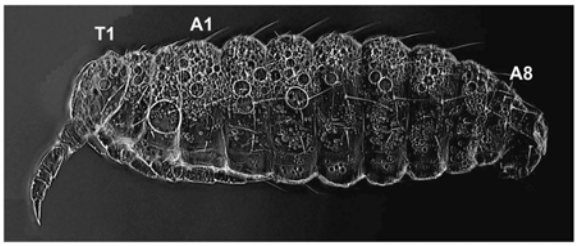
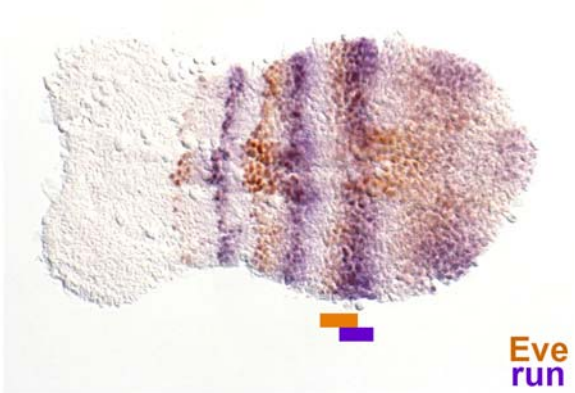


Figure 1.7 Double staining of Tc-Eve and *Tc-run*.

The anterior region of a primary *Tc-run* (purple) stripe overlaps the posterior 50% of a primary Tc-Eve (gold) stripe.



CHAPTER 2 - 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*

Chong Pyo Choe and Susan J. Brown*

Division of Biology, Kansas State University, Manhattan, KS USA

***Corresponding author**: Susan J. Brown

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

Email: sjbrown@ksu.edu, Phone: (785) 532-3935, Fax: (785) 532-6653

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.

Key words: *paired*; *sloppy-paired*; segmentation; pair-rule gene; *Tribolium castaneum*
Word counts: 194 words

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, 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., 2001). 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 Arial 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, *slp 1* 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 *slp 1* 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). *slp 1* 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 – 48 hour 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 X injection buffer or 1µg/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 hours for six 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, 0-24 hour 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 seconds every 10 minutes for 1 hour. 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 50ul of squish buffer (Gloor et al., 1993) and incubating it with proteinase K for 1 hour at 25°C. 2ul 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. 2.1A). 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. 2.1C). 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. 2.2A). 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. 2.2 A, 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. 2.2C). 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. 2.2D). 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. 2.2E and summarized in Fig. 2.7A). 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. 2.2E). 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. 2.2 E-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. 2.2I). 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 thoracic segments always displayed clear pair-rule phenotypes (Figs. 2.3 B, C). However, the series of *Tc-prd*^{RNAi} embryos showed variation in the number of abdominal segments affected (Figs. 2.3 B, C, compare to 2.3A). Most *Tc-prd*^{RNAi} embryos (90.2%)

were strongly affected and displayed complete pair-rule phenotypes containing only 4 or 5 abdominal segments (Fig. 2.3B) while weak *Tc-prd*^{RNAi} embryos (8.7%), showed deletion of 3 or fewer abdominal segments (Fig. 2.3C), 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. 2.3 F, H, compare to 2.3E). 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. 2.3G). 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. 2.7B). 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. 2.7 A, 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. 2.7B), 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. 2.4A). Soon thereafter this stripe is limited ventrally in the presumptive head lobes of the future germ rudiment (Fig. 2.4B), in the regions that give rise to the antennae (Fig. 2.4J). Before the germ rudiment condenses, a new *Tc-slp* stripe appears in the blastoderm (arrowhead in Fig. 2.4C). Double staining with anti-En antibody indicates that this second stripe is expressed in the presumptive mandibular segment (Fig. 2.4E). Just after the germband forms, a narrow *Tc-slp* stripe appears in the presumptive maxillary segment (arrowhead

in Fig. 2.4D). Then a strong stripe (arrowheads in Figs. 2.4 E, F) in the first thoracic segment appears prior to a weak narrower stripe in the labial segment (arrow Fig. 2.4F). During germband elongation, pairs of *Tc-slp* stripes appear in the anterior region of the growth zone (Figs. 2.4 G-K). The anterior stripe (arrows in Figs. 2.4 G-K) is narrower and weaker than the posterior stripe (arrowheads in Figs. 2.4 G-K). As they develop, each *Tc-slp* stripe overlaps the anterior row of cells in a Tc-En stripe (Figs. 2.4 G-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. 2.7A. 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 slp 1* 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. 2.5 B, C, compare to 2.5A) 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. 2.4 B-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. 2.5 B, C, compare to 2.5A).

The most severe *Tc-slp*^{RNAi} embryos (8.3%) displayed a compact segmental phenotype with 4 asymmetrically incomplete segments (Fig. 2.5B; see 4 segments (white dots) on one side and 2 broad segments (white arrowheads) 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. 2.5C).

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. 2.5G, compare to 2.5E). 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. 2.5H). 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. 2.7B). 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) 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. 2.7B), 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 Fig. 2.5G and 2.5E) 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. 2.5F, compare to 2.5B). 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. 2.7 A, 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. 2.3 B, C, 2.5 B, 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. 2.6C, compare to 2.6 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 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. 2.1B). Alignment of the protein sequences indicated that this region is not highly conserved between *Drosophila* and *Tribolium* (asterisk in Fig. 2.1A), making it difficult to imagine how this missense mutation may cause the *scy* phenotype. However, two *Drosophila prd* alleles, prdX3 and prdIIN 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. 2.3D). 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. 2.1D). 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. 2.5D) 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.

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. 2.7A), 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 co-expressed 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. 2.7A). *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 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. 2.8,

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 didn't diverge much at the sequence level (*Dm-slp*2). 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.

Acknowledgements

We thank M. Klingler for the *scy* and *icy* mutants. mAbs 4D9 and 2B8 developed by N.H. Patel were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. We thank members of the lab for helpful comments and discussions. This work was supported by a grant from the National Institutes of Health (R01-HD29594).

References

Anderson, P., 1995. Mutagenesis. Methods Cell Biol. 48, 31-58.

Baumgartner, S., Noll, M., 1990. Network of interactions among pair-rule genes regulating paired expression during primordial segmentation of *Drosophila*. Mech.Dev. 33, 1-18.

Benedyk, M. J., Mullen, J. R., DiNardo, S., 1994. *odd-paired*: a zinc finger pair-rule protein required for the timely activation of *engrailed* and *wingless* in *Drosophila* embryos. Genes Dev. 8, 105-117.

Bennett, R. L., Brown, S. J., Denell, R. E., 1999. Molecular and genetic analysis of the *Tribolium Ultrabithorax* ortholog, *Ultrathorax*. Dev.Genes Evol. 209, 608-619.

Bertuccioli, C., Fasano, L., Jun, S., Wang, S., Sheng, G., Desplan, C., 1996. In vivo requirement for the paired domain and homeodomain of the *paired* segmentation gene product. Development 122, 2673-2685.

Brown, S., Holtzman, S., Kaufman, T., Denell, R., 1999a. Characterization of the *Tribolium Deformed* ortholog and its ability to directly regulate *Deformed* target genes in the rescue of a *Drosophila Deformed* null mutant. Dev.Genes Evol. 209, 389-398.

Brown, S. J., Hilgenfeld, R. B., Denell, R. E., 1994. The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. Proc.Natl.Acad.Sci.U.S.A 91, 12922-12926.

Brown, S. J., Mahaffey, J. P., Lorenzen, M. D., Denell, R. E., Mahaffey, J. W., 1999b. Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. Evol.Dev. 1, 11-15.

Brown, S. J., Parrish, J. K., Beeman, R. W., Denell, R. E., 1997. Molecular characterization and embryonic expression of the *even-skipped* ortholog of *Tribolium castaneum*. Mech.Dev. 61, 165-173.

Bucher, G., Klingler, M., 2004. Divergent segmentation mechanism in the short germ insect *Tribolium* revealed by *giant* expression and function. Development 131, 1729-1740.

Bucher, G., Scholten, J., Klingler, M., 2002. Parental RNAi in *Tribolium* (Coleoptera). Curr.Biol. 12, R85-R86.

Cadigan, K. M., Grossniklaus, U., Gehring, W. J., 1994a. Functional redundancy: the respective roles of the two *sloppy paired* genes in *Drosophila* segmentation. Proc.Natl.Acad.Sci.U.S.A 91, 6324-6328.

Cadigan, K. M., Grossniklaus, U., Gehring, W. J., 1994b. Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments. Genes Dev. 8, 899-913.

Cerny, A. C., Bucher, G., Schroder, R., Klingler, M., 2005. Breakdown of abdominal patterning in the *Tribolium Kruppel* mutant *jaws*. Development 132, 5353-5363.

Chipman, A. D., Arthur, W., Akam, M., 2004. A double segment periodicity underlies segment generation in centipede development. Curr.Biol. 14, 1250-1255.

Choe, C. P., Miller, S. C., Brown, S. J., 2006. A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. Proc Natl Acad Sci U S A 103, 6560-4.

Coulter, D. E., Wieschaus, E., 1988. Gene activities and segmental patterning in *Drosophila*: analysis of *odd-skipped* and pair-rule double mutants. Genes Dev. 2, 1812-1823.

Curtis, C. D., Brisson, J. A., DeCamillis, M. A., Shippy, T. D., Brown, S. J., Denell, R. E., 2001. Molecular characterization of *Cephalothorax*, the *Tribolium* ortholog of *Sex combs reduced*. Genesis. 30, 12-20.

Damen, W. G., Hausdorf, M., Seyfarth, E. A., Tautz, D., 1998. A conserved mode of head segmentation in arthropods revealed by the expression pattern of Hox genes in a spider. Proc.Natl.Acad.Sci.U.S.A 95, 10665-10670.

Damen, W. G., Janssen, R., Prpic, N. M., 2005. Pair rule gene orthologs in spider segmentation. Evol.Dev. 7, 618-628.

Davis, G. K., Jaramillo, C. A., Patel, N. H., 2001. Pax group III genes and the evolution of insect pair-rule patterning. Development 128, 3445-3458.

Davis, G. K., Patel, N. H., 2002. Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu.Rev.Entomol. 47, 669-699.

Dawes, R., Dawson, I., Falciani, F., Tear, G., Akam, M., 1994. *Dax*, a locust Hox gene related to *fushi-tarazu* but showing no pair-rule expression. Development 120, 1561-1572.

Dearden, P. K., Donly, C., Grbic, M., 2002. Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite *Tetranychus urticae*. Development 129, 5461-5472.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S., Noll, M., 1986. Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. Cell 47, 735-746.

Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M., Engels, W. R., 1993. Type I repressors of P element mobility. Genetics 135, 81–95.

Grossniklaus, U., Cadigan, K. M., Gehring, W. J., 1994. Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head. Development 120, 3155-3171.

Grossniklaus, U., Pearson, R. K., Gehring, W. J., 1992. The *Drosophila sloppy paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. Genes Dev. 6, 1030-1051.

Ingham, P. W., 1988. The molecular genetics of embryonic pattern formation in *Drosophila*. Nature 335, 25-34.

Ingham, P. W., Baker, N. E., Martinez-Arias, A., 1988. Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped*. Nature 331, 73-75.

Ingham, P. W., Martinez-Arias, A., 1986. The correct activation of Antennapedia and bithorax complex genes requires the *fushi tarazu* gene. Nature 324, 592-597.

Jaynes, J. B., Fujioka, M., 2004. Drawing lines in the sand: *even-skipped* et al. and parasegment boundaries. Dev. Biol. 269, 609-622.

Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E., Noll, M., 1986. Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. Nature 321, 493-499.

Liu, P. Z., Kaufman, T. C. 2005., *even-skipped* is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect. Development 132, 2081-2092.

Maderspacher, F., Bucher, G., Klingler, M., 1998. Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. Dev.Genes Evol. 208, 558-568.

Martinez Arias, A., Baker, N. E., Ingham, P. W., 1988. Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. Development 103, 157-170.

Mito, T., Sarashina, I., Zhang, H., Iwahashi, A., Okamoto, H., Miyawaki, K., Shinmyo, Y., Ohuchi, H., Noji, S., 2005. Non-canonical functions of *hunchback* in segment patterning of the intermediate germ cricket *Gryllus bimaculatus*. Development 132, 2069-2079.

Morrissey, D., Askew, D., Raj, L., Weir, M., 1991. Functional dissection of the *paired* segmentation gene in *Drosophila* embryos. Genes Dev. 5, 1684-1696.

Nasiadka, A., Dietrich, B. H., Krause, H. M., 2002. Anterior-posterior patterning in the *Drosophila* embryo. Adv. Dev. Biol. Biochem. 12, 155-204.

Niessing, D., Rivera-Pomar, R., La Rosee, A., Hader, T., Schock, F., Purnell, B. A., Jackle, H., 1997. A cascade of transcriptional control leading to axis determination in *Drosophila*. J.Cell Physiol 173, 162-167.

Nulsen, C., Nagy, L. M., 1999. The role of *wingless* in the development of multibranched crustacean limbs. Dev.Genes Evol. 209, 340-348.

Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. Nature 287, 795-801.

Osborne, P. W., Dearden, P. K., 2005. Expression of Pax group III genes in the honeybee (*Apis mellifera*). Dev.Genes Evol. 215, 499-508.

Patel, N. H., Ball, E. E., Goodman, C. S., 1992. Changing role of *even-skipped* during the evolution of insect pattern formation. Nature 357, 339-342.

Patel, N. H., Condron, B. G., Zinn, K., 1994. Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. Nature 367, 429-434.

Patel, N. H., Hayward, D. C., Lall, S., Pirkl, N. R., DiPietro, D., Ball, E. E., 2001. Grasshopper *hunchback* expression reveals conserved and novel aspects of axis formation and segmentation. Development 128, 3459-3472.

Pourquie, O., 2003. The segmentation clock: converting embryonic time into spatial pattern. Science 301, 328-330.

Schoppmeier, M., Damen, W. G., 2005. Expression of Pax group III genes suggests a single-segmental periodicity for opisthosomal segment patterning in the spider *Cupiennius salei*. Evol.Dev. 7, 160-169.

Sommer, R. J., Tautz, D., 1993. Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). Nature 361, 448-450.

Stollewerk, A., Schoppmeier, M., Damen, W. G., 2003. Involvement of *Notch* and *Delta* genes in spider segmentation. Nature 423, 863-865.

Stuart, J. J., Brown, S. J., Beeman, R. W., Denell, R. E., 1991. A deficiency of the homeotic complex of the beetle *Tribolium*. Nature 350, 72-4.

Thompson, J. D., Higgins, D. G., Gibson, T. J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Figures

Figure 2.1 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.

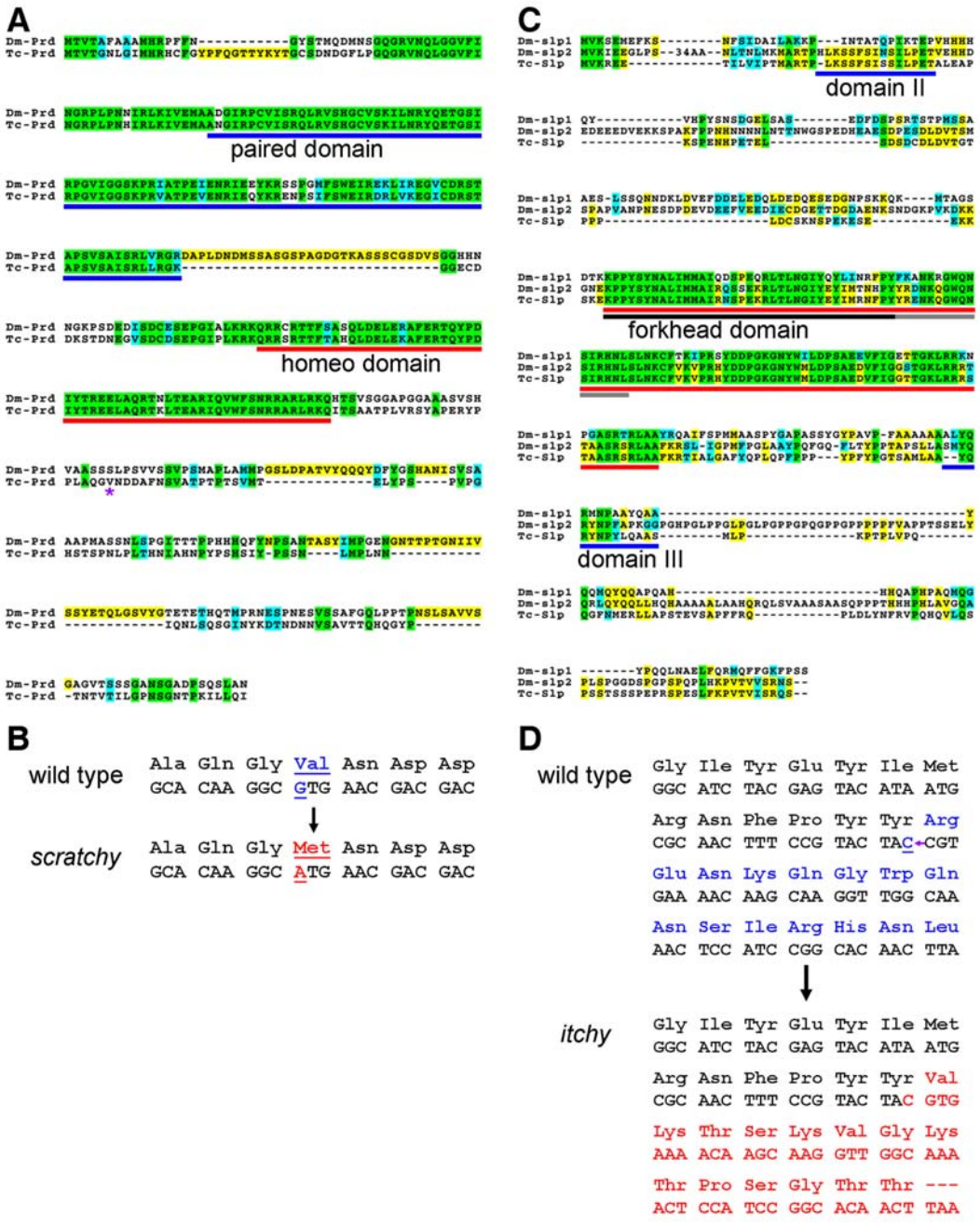

A
paired domain
homeo domain
C
domain II
forkhead domain
domain III
B
wild type
Ala Gln Gly Val Asn Asp Asp
GCA CAA GGC GTG AAC GAC GAC
scratchy
Ala Gln Gly Met Asn Asp Asp
GCA CAA GGC ATG AAC GAC GAC
D
wild type
Gly Ile Tyr Glu Tyr Ile Met
GGC ATC TAC GAG TAC ATA ATG
Arg Asn Phe Pro Tyr Tyr Arg
CGC AAC TTT CCG TAC TAC CGT
Glu Asn Lys Gln Gly Trp Gln
GAA AAC AAG CAA GGT TGG CAA
Asn Ser Ile Arg His Asn Leu
AAC TCC ATC CGG CAC AAC TTA
itchy
Gly Ile Tyr Glu Tyr Ile Met
GGC ATC TAC GAG TAC ATA ATG
Arg Asn Phe Pro Tyr Tyr Val
CGC AAC TTT CCG TAC TAC GTG
Lys Thr Ser Lys Val Gly Lys
AAA ACA AGC AAG GTT GGC AAA
Thr Pro Ser Gly Thr Thr ---
ACT CCA TCC GGC ACA ACT TAA

Figure 2.2 Expression of *Tc-prd* in *Tribolium* embryos undergoing segmentation. (A-C and E-I) are stained with *Tc-prd* riboprobe (purple) and Anti-En antibody (punctate, 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.

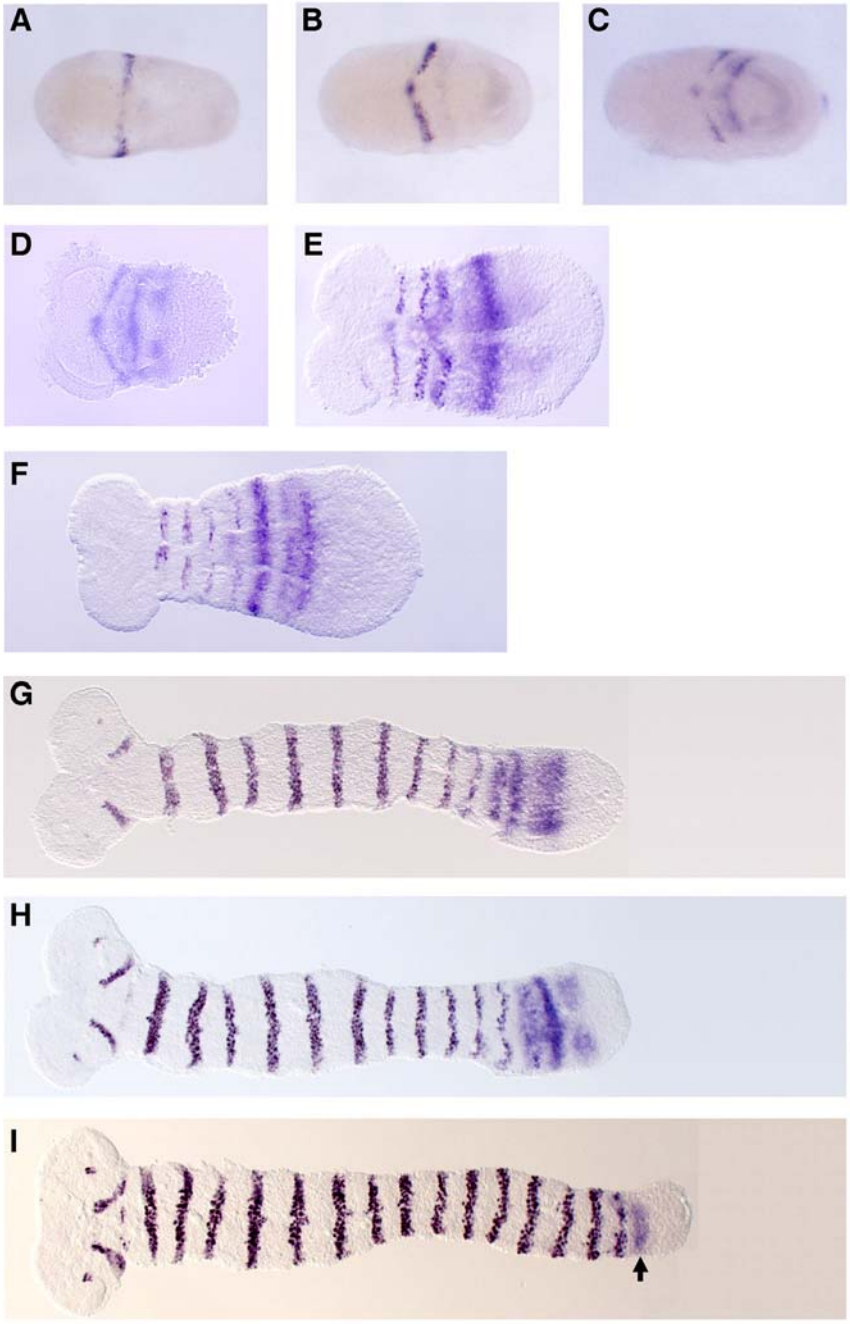

A
B
C
D
E
F
G
H
I

Figure 2.3 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, arrowheads; 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 (punctate, brown spots) and *Tc-prd* (purple). The defective odd-numbered En stripes are marked with arrowheads 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 (punctate, 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 (punctate, 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 (arrowhead) 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.

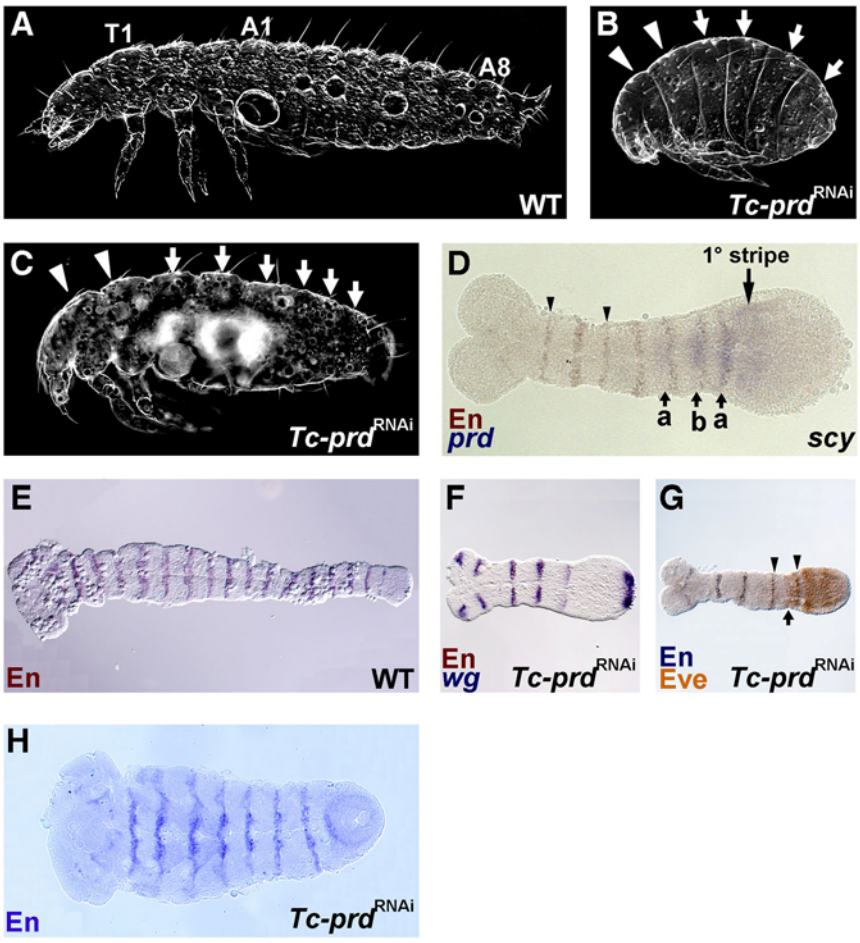

A
T1
A1
A8
WT
B
Tc-prd RNAi
C
Tc-prd RNAi
D
1° stripe
a
b
a
En
prd
scy
E
En
WT
F
En
wg
Tc-prd RNAi
G
En
Eve
Tc-prd RNAi
H
En
Tc-prd RNAi

Figure 2.4 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 (punctate, brown spots). (G-K) Primary *Tc-slp* stripes, arrowhead; Secondary Tc-slp stripes, arrow. (A-D) Blastoderm stage. (E-K) Germband stages. (A) The first *Tc-slp* stripe (arrowhead) appears de novo in the anterior region of the embryo (future head lobes). (B)This stripe (arrowhead) is split by the mesoderm at the ventral midline. (C)The second *Tc-slp* stripe (arrowhead) appears first in the ectoderm and then in the mesoderm (D). The third *Tc-slp* stripe (arrowhead in 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 (arrowhead) appears as two spots flanking the mesoderm. (F) A narrow and weak fourth *Tc-slp* stripe (arrow) appears anterior to the fifth stripe (arrowhead). (G) A pair of *Tc-slp* stripes (arrow and arrowhead) 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; arrowhead) is strong. (H-K) The next pair of *Tc-slp* stripes (arrow and arrowhead) 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.

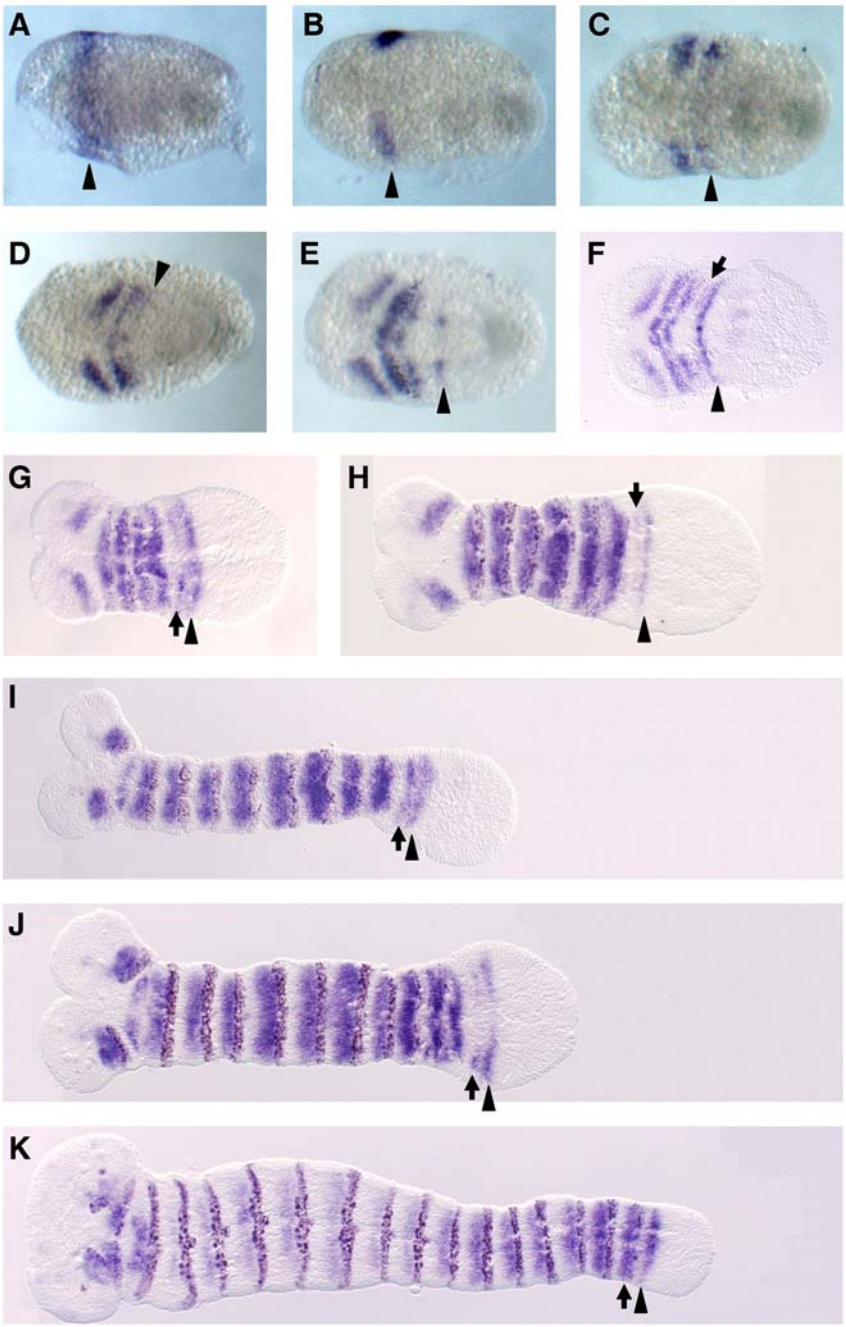

A
B
C
D
E
F
G
H
I
J
K

Figure 2.5 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 (arrowheads) and four segments on the other (white dots). (C)The intermediate phenotype of *Tc-slp*^{RNAi} produces embryos containing T2 (arrowhead) 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 (punctate, 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 (arrowhead) 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 (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 (punctate, dark blue spots) and anti-Eve antibodies (punctate, brown spots). In this germband, even-numbered Tc-En stripes, which were coexpressed with Tc-Eve b stripes (arrowhead) 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.

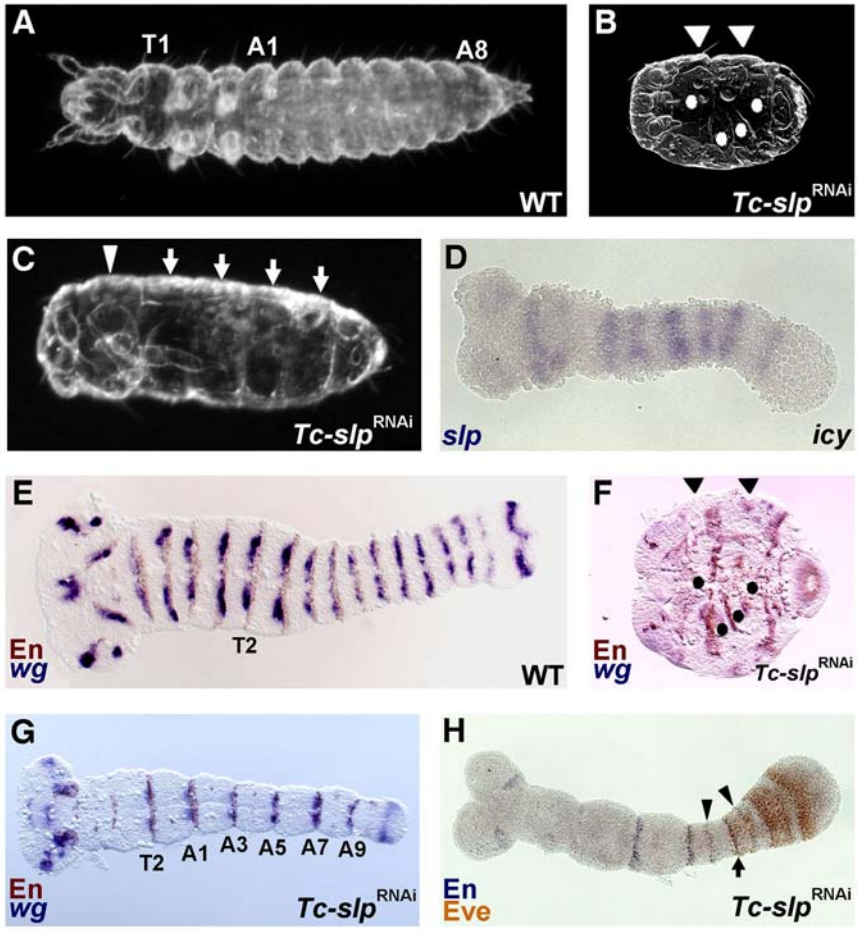


Figure 2.6 *Tc-Dfd* expression in *Tribolium* germband embryos.

(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 (punctate, 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.

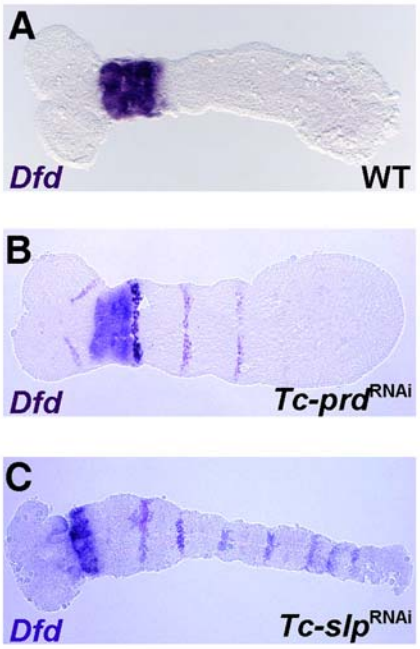


Figure 2.7 Summary of secondary pair-rule gene expression relative to other segmentation genes in *Tribolium* and the effects of secondary pair-rule gene mutations or RNAi on the expression of *en* and *wg* in *Drosophila* and *Tribolium*. (A) Pair-rule (upper) and segment polarity (lower) expression 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.

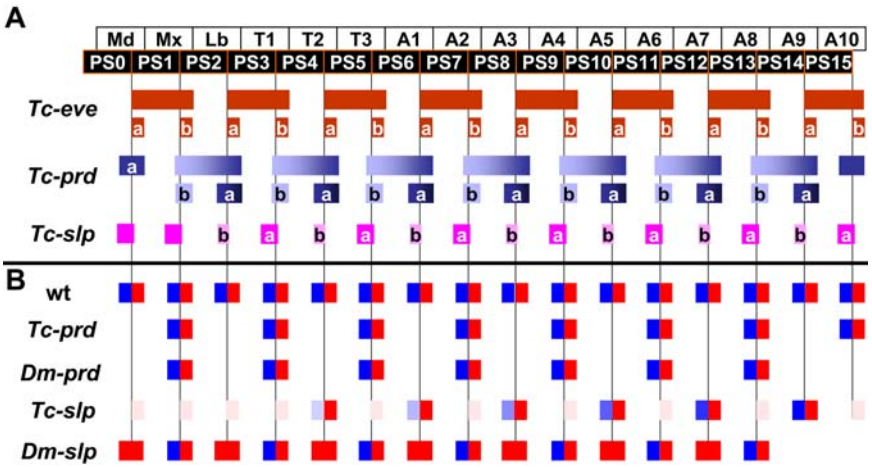
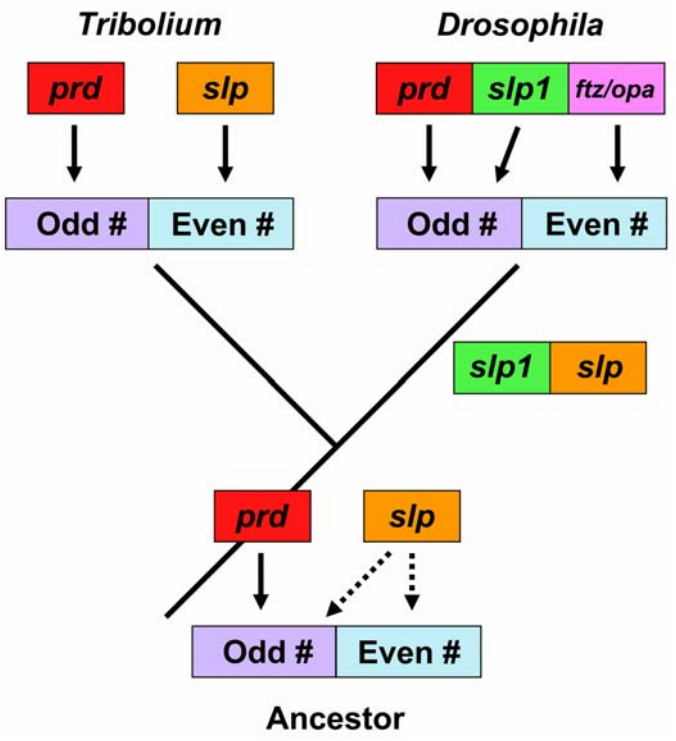


Figure 2.8 Comparison of secondary pair-rule gene functions in *Drosophila* and *Tribolium* in an evolutionary context.

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.



CHAPTER 3 - Redeployment of primary pair-rule genes for the regulation of *engrailed* and *wingless* in *Tribolium* segmentation

Chong Pyo Choe and Susan J. Brown*

Division of Biology, Kansas State University, Manhattan, KS, USA

***Correspondence**: Susan J. Brown

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

Email: sjbrown@ksu.edu, Phone: (785) 532-3935, Fax: (785) 532-6653

Abbreviations: *engrailed* (*en*), *wingless* (*wg*), *even-skipped* (*eve*), *runt* (*run*), *odd-skipped* (*odd*), *paired* (*prd*), *sloppy-paired* (*slp*)

Abstract

In the long-germ insect *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. In *Tribolium*, the homologs of *Drosophila* secondary pair-rule genes *fushi tarazu* and *odd-paired* are not required for proper segmentation. Nonetheless, *Tc-en* and *Tc-wg* expression at parasegmental boundaries is conserved. Thus, it remains to be determined how *Tribolium* pair-rule genes regulate segment polarity genes. We used RNAi to examine the results of expressing one or two pair-rule genes in the absence of the other known pair-rule genes. We found that the primary pair-rule genes, *Tribolium even-skipped* (*Tc-eve*) and *runt* (*Tc-run*), in combination with the secondary pair-rule genes *Tribolium paired* and *sloppy-paired*, regulate expression of *Tc-en* and *Tc-wg* at the parasegmental boundaries. The primary stripes of *Tc-eve* and *Tc-run* resolve into secondary stripes that appear to provide secondary function directly to regulate *Tc-en* and *Tc-wg*, accounting for a seemingly smaller complement of pair-rule genes in *Tribolium* relative to *Drosophila*. Alternatively, *Tc-eve* and *Tc-run* may control additional, as yet unidentified, secondary pair-rule genes that provide secondary function to regulate *Tc-en* and *Tc-wg*. It has previously been suggested from computation modeling that the developmental module of segment polarity genes is likely to be resistant to variations in regulatory inputs. Our results provide the first experimental evidence for such evolutionary variation in pair-rule gene regulation of segment polarity genes.

Introduction

Detailed genetic and molecular analysis in the long-germ insect *Drosophila* has revealed a well-organized segmentation hierarchy of maternal, gap, pair-rule and segment polarity genes. Through the segmentation hierarchy, *Drosophila* embryos are subdivided along the anterior-posterior axis into narrow regions and finally divided into reiterated segments (1). In this segmentation hierarchy, 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 other short-germ insects and arthropods. Interestingly, despite the conserved segmental expression patterns of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) at parasegmental boundaries, accumulating genetic and molecular evidence from other insects and arthropods suggest that the functions of segmentation genes upstream of the segment polarity genes have diverged considerably (1). However, it remains to be determined how divergent functions of upstream factors in the segmentation hierarchy ultimately generate conserved expression patterns of *en* and *wg* to define segments in nondrosophilid insects.

Previously, we described a primary pair-rule gene circuit comprising *even-skipped* (*eve*), *runt* (*run*), and *odd-skipped* (*odd*) that sequentially prepatterns a two-

segment wide region (2), 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, in the short-germ insect *Tribolium* (2). Furthermore, *hairy* (*h*), *fushi tarazu* (*ftz*) and *odd-paired* (*opa*), which are key activators of *en* and *wg* expression at the anterior boundary of even-numbered parasegments in *Drosophila* (3-6), do not appear to function as segmentation genes in *Tribolium* (2, 7). Despite these differences in pair-rule gene functions between *Drosophila* and *Tribolium*, *Tc-en* and *Tc-wg* stripes are expressed at the parasegmental boundaries similar to stripes of *en* and *wg* in *Drosophila* (8, 9, and Fig. 3.1*a*), indicating that *Tribolium* pair-rule genes regulate segment polarity genes differently from their *Drosophila* homologs.

In order to understand how *Tribolium* pair-rule genes regulate the 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. 3.1*a*), *Tc-eve* is required to activate *Tc-run*, which is required to activate *Tc-odd*, which is then required to repress *Tc-eve*, sequentially generating primary stripes of *Tc-eve* (2). In addition, the secondary pair-rule genes, *Tc-prd* and *Tc-slp*, which occupy parallel positions in this network, are repressed by *Tc-run* (2). 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 (2). 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.

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 are

misexpressed. We found that combinations of pair-rule genes different from those in *Drosophila* are required in *Tribolium* to regulate the segment polarity genes at each parasegmental boundary. In *Tribolium*, the primary stripes of *Tc-eve* and *Tc-run* resolve into secondary stripes after prepatterning a two-segment wide region and, in combination with *Tc-prd* or *Tc-slp*, regulate *Tc-en* and *Tc-wg*. Among the homologs of *Drosophila* secondary pair-rule genes, only *Tc-prd* and *Tc-slp* provide functions essential for segmentation in *Tribolium*. Our results suggest that primary pair-rule genes provide additional secondary functions directly, or via regulation of as yet unidentified secondary pair-rule genes to regulate segment polarity genes. While other studies have implicated regulatory differences, we provide experimental evidence of evolutionary variation in pair-rule gene regulation of segment polarity genes.

Results

Regulation of *Tc-en* and *Tc-wg* by *Tribolium* primary pair-rule genes

Previously we reported that *Tribolium* primary pair-rule genes are important to prepattern a two-segment wide region 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* in *Tribolium* (2, 10). Interestingly, after two-segment wide regions are prepatterned, the primary stripes of the primary pair-rule genes resolve into narrow secondary stripes at the parasegmental boundaries, in cells that will express *Tc-en* (2, 11, 12). Therefore, it is possible 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 (Fig. 3.1). In strong *Tc-eve*^{RNAi} embryos, the striped expression of *Tc-en* was abolished while *Tc-wg* was expressed weakly in a broad region in the middle of the embryo instead of in stripes (Fig. 3.1*c*, and Table 3.1). Similarly, the expression of *Tc-en* was lost (2), and the striped expression of *Tc-wg* was replaced by a broad domain in *Tc-run*^{RNAi} embryos (Fig. 3.1*d*, and Table 3.1). Together, these results suggest that *Tc-eve* and *Tc-run* are required for the activation of *Tc-en* and for the repression of *Tc-wg*. In contrast to *Tc-eve*^{RNAi} or *Tc-run*^{RNAi}, strong *Tc-odd*^{RNAi} caused expanded but weak *Tc-en* expression and complete abolishment of *Tc-wg* expression (Fig. 3.1*e*, and Table 3.1), indicating that *Tc-odd* is required for the activation of *Tc-wg* and for the repression of *Tc-en*. However, we have previously shown that *Tc-eve*, *Tc-run* and *Tc-odd* are primary pair-rule genes that regulate expression of the secondary pair-rule genes *Tc-prd* and *Tc-slp* (2). 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.

Analysis of genetic interactions affecting *Tc-en* and *Tc-wg* expression at the anterior boundary of odd-numbered parasegments

In wild type embryos, *Tc-eve* and *Tc-prd* are expressed in secondary stripes that are coincident with stripes of *Tc-en* in odd-numbered parasegments (10-12, and Fig. 3.4*a*). The spatial and temporal relationship between these secondary stripes (*Tc-eve* a and *Tc-prd* a) and *Tc-en* in odd-numbered parasegments suggests that one or both are required to activate *Tc-en* stripes in odd-numbered parasegments. Furthermore, the selective elimination of *Tc-en* stripes in odd-numbered parasegments by *Tc-prd*^{RNAi} reveals that *Tc-prd* is a key activator of the *Tc-en* stripes (2, and Fig. 3.1*f*). To address whether *Tc-prd* alone is sufficient to activate *Tc-en*, we performed double RNAi with *Tc-eve* and *Tc-slp*, which results in ectopic *Tc-prd* (Fig. 3.2*c*) in the absence of *Tc-eve*, *Tc-run*, *Tc-odd*, and *Tc-slp* expression (Fig. 3.2*d*, Fig. 3.5 *a*, *b*, which is published as supporting information on the PNAS web site). If *Tc-prd* is sufficient to activate *Tc-en* expression, then *Tc-en* should be initiated in these double RNAi embryos. However, *Tc-en* was not initiated in these embryos (Fig. 3.2*a*) suggesting that *Tc-prd* alone is not sufficient to activate *Tc-en* expression (Table 3.1).

We have previously shown that *Tc-eve* is expressed normally in the odd-numbered parasegments of *Tc-prd*^{RNAi} embryos, but *Tc-en* stripes are not initiated (2), indicating that *Tc-eve* is also not sufficient to activate *Tc-en*. To determine if together *Tc-prd* and *Tc-eve* are sufficient to activate *Tc-en*, we performed double RNAi with *Tc-run* and *Tc-slp*, which resulted in ectopic expression of *Tc-prd* and *Tc-eve* (Fig. 3.2 *g*, *h*), in the absence of *Tc-run*, *Tc-odd*, and *Tc-slp* expression (Fig. 3.5 *c*, *d*). In the double RNAi embryos, *Tc-en* was expressed broadly but weakly (Fig. 3.2*e*), indicating that the

combination of *Tc-prd* and *Tc-eve* is sufficient to activate *Tc-en* (compare Fig. 3.2 *e*, *g*, *h* to Fig. 3.2 *a*, *c*, *d*, respectively, Table 3.1).

On the other side of the parasegmental boundary, in the even-numbered parasegments of wild type embryos, secondary stripes of *Tc-prd* and *Tc-slp* are expressed in the cells that express *Tc-wg* (10), suggesting that one or both regulate the expression of *Tc-wg* here. Furthermore, in *Tc-prd*^{RNAi} embryos initiation of these *Tc-wg* stripes is completely abolished, indicating that *Tc-prd* is required to activate them (10, and Fig. 3.1*f*). In contrast, these *Tc-wg* stripes are initiated normally and then fade in *Tc-slp*^{RNAi} embryos, indicating that *Tc-slp* is not essential to activate but is required to maintain them (10, and Fig. 3.1*g*). To determine if *Tc-prd* is sufficient to activate *Tc-wg* we examined *Tc-eve*, *Tc-slp* double RNAi embryos, where *Tc-prd* was ectopically expressed (Fig. 3.2*c*), for the expression of *Tc-wg*. If *Tc-prd* is sufficient to activate *Tc-wg*, then *Tc-wg* should be expressed, and indeed it is (Fig. 3.2*b*). Because *Tc-run*, *Tc-odd* and *Tc-slp* are not expressed in these embryos (Fig. 3.5 *a*, *b*), it appears that *Tc-prd* alone is able to activate *Tc-wg* (Table 3.1). Taken together with the observation that *Tc-slp* is not required to activate these *Tc-wg* stripes, we suggest that *Tc-prd* is sufficient to activate *Tc-wg* stripes in even-numbered parasegments in wild type embryos.

Thus far we have suggested that *Tc-en* in odd-numbered parasegments is activated by the combination of *Tc-eve* and *Tc-prd* whereas the adjacent *Tc-wg* stripes in even-numbered parasegments are activated by *Tc-prd* alone in wild type embryos. However, if *Tc-prd* is required to activate *Tc-en* and *Tc-wg* expression, how do cells expressing *Tc-prd* selectively express *Tc-en* or *Tc-wg* on either side of the

parasegmental boundary? To determine if *Tc-eve*, which is coexpressed with *Tc-en* at the parasegmental boundary, suppresses *Tc-prd* activation of *Tc-wg*, we examined *Tc-run*, *Tc-slp* double RNAi embryos for the expression of *Tc-wg*. In these embryos, *Tc-prd* and *Tc-eve* were expressed (Fig. 3.2 *g*, *h*) in the absence of *Tc-run*, *Tc-odd* and *Tc-slp* (Fig. 3.5 *c*, *d*). We found that *Tc-wg* is not activated in these double RNAi embryos (Fig. 3.2*f*), indicating *Tc-eve* suppresses the *Tc-prd* activation of *Tc-wg* in odd-numbered parasegments (compare Fig. 3.2 *f-h* to Fig. 3.2 *b-d*, respectively, Table 3.1).

In summary, we suggest that the *Tc-wg* stripe in even-numbered parasegments is initiated by *Tc-prd*, while the adjacent *Tc-en* stripe in odd-numbered parasegments is activated by the combination of *Tc-prd* and *Tc-eve*, which is also required to repress *Tc-wg* here (Fig. 3.4*a*).

Analysis of genetic interactions affecting *Tc-en* and *Tc-wg* expression at the anterior boundary of even-numbered parasegments

In wild type embryos, *Tc-slp* is expressed in stripes that alternate in intensity (10). The stronger (primary) stripes are expressed in cells that will express *Tc-wg* in odd-numbered parasegments (10). This temporal and spatial relationship suggests that *Tc-slp* may be required and/or sufficient to activate these *Tc-wg* stripes. Furthermore, the selective elimination of these *Tc-wg* stripes in *Tc-slp*^{RNAi} embryos revealed that *Tc-slp* is a key activator of *Tc-wg* here (10, and Fig. 3.1*g*). To address whether *Tc-slp* alone is sufficient to activate *Tc-wg*, we performed *Tc-eve*, *Tc-prd* double RNAi. As expected, *Tc-slp* was expressed in these embryos (Fig. 3.3*b*) whereas *Tc-eve*, *Tc-run*, *Tc-odd* and *Tc-prd* were not (Fig. 3.3*c*, Fig. 3.6 *a*, *b*, which is published as supporting information on

the PNAS web site). If *Tc-slp* is sufficient to activate *Tc-wg*, we expected to see *Tc-wg* expression in these embryos. Indeed, *Tc-wg* was expressed broadly in these double RNAi embryos (Fig. 3.3*a*), which suggests that *Tc-slp* is sufficient to activate *Tc-wg* (Table 3.1).

However, previously we reported that the very posterior rows of cells expressing *Tc-slp* overlaps the anterior rows of cells of the *Tc-en* stripes in the adjacent even-numbered parasegments (10). If *Tc-slp* is sufficient to activate *Tc-wg* stripes, why do these cells, which express *Tc-slp*, express *Tc-en* instead of *Tc-wg*? To determine whether *Tc-eve*, which is coexpressed with *Tc-en*, is required to repress *Tc-slp* activation of *Tc-wg* in even-numbered parasegments, we examined *Tc-run*, *Tc-prd* double RNAi embryos (Fig. 3.6 *c*, *d*) for the expression of *Tc-wg*. In these embryos *Tc-slp* and *Tc-eve* are expressed ectopically (Fig. 3.3 *e*, *f*). *Tc-wg* is expressed broadly when only *Tc-slp* is expressed (Fig. 3.3*a*). If *Tc-eve* suppresses the activation of *Tc-wg* by *Tc-slp* we expect *Tc-wg* expression to be eliminated or severely reduced in the double RNAi embryos. Indeed, we found that the expression of *Tc-wg* was limited to a narrow region in the middle of the double RNAi embryos (Fig. 3.3*d*), which supports the idea that *Tc-eve* is required to restrict *Tc-slp* activation of *Tc-wg* to odd-numbered parasegments (compare Fig. 3.3 *d-f* to Fig. 3.3 *a-c*, respectively, Table 3.1).

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 express *Tc-en* (2, 10-12). 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. However, *Tc-prd*^{RNAi} revealed

that *Tc-prd* is not required to activate these *Tc-en* stripes (2, and Fig. 3.1*f*). Furthermore, *Tc-odd* is required to repress *Tc-en* rather than to activate it (Fig. 3.1*e*). To determine whether *Tc-eve* and *Tc-run* are sufficient for the activation of these *Tc-en* stripes we examined the expression of *Tc-en* in *Tc-odd*^{RNAi} embryos, in which *Tc-eve* and *Tc-run* are ectopically expressed in the absence of *Tc-prd* and *Tc-slp* in these embryos (2, and Fig. 3.3 *h*, *i*). *Tc-en* in situ hybridization in the *Tc-odd*^{RNAi} embryos revealed strong, broad *Tc-en* expression (Fig. 3.3*g*), which suggests that either *Tc-eve*, *Tc-run* or both are required to activate expression of *Tc-en* (Table 3.1).

To determine if *Tc-eve* alone is sufficient to activate *Tc-en*, 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 (2), we expected that only *Tc-eve* would be expressed in the triple RNAi embryos. Indeed, *Tc-eve* was expressed in the absence of the other pair-rule genes in the triple RNAi embryos (Fig. 3.3 *k*, *l* and Fig. 3.6 *e*, *f*). If *Tc-eve* is sufficient to activate *Tc-en*, we expected that *Tc-en* would be expressed in the triple RNAi embryos. However, it was not (Fig. 3.3*j*), indicating that although *Tc-eve* is required, it is not sufficient to activate *Tc-en* (Table 3.1). 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 others to test whether *Tc-run* is sufficient to activate *Tc-en*. While overexpression of *Tc-run* might show whether *Tc-run* is sufficient to activate *Tc-en*, 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, strongly suggest that *Tc-run* is required to activate *Tc-en* without the additional input of *Tc-prd* and *Tc-slp*. Taken together with the coexpression of *Tc-eve* and *Tc-run* with *Tc-en*

stripes in even-numbered parasegments in wild type embryos, we suggest that *Tc-eve* b and the secondary *Tc-run* stripes are required to activate *Tc-en* in even-numbered parasegments.

In summary, we suggest that *Tc-wg* in odd-numbered parasegments is activated by *Tc-slp*, and that *Tc-en* in adjacent even-numbered parasegments is activated by the combination of *Tc-eve* and *Tc-run* (Fig. 3.4*a*).

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, we provide some insights into the genetic mechanism by which *Tribolium* pair-rule genes regulate the conserved striped expression of *Tc-en* and *Tc-wg* at parasegmental boundaries. Furthermore, it is now possible to compare the regulation of *en* and *wg* by pair-rule genes between *Drosophila* and *Tribolium* to understand the evolution of these regulatory functions in the lineages leading to *Drosophila* and *Tribolium*. Below we describe a model of the regulation of *Tc-en* and *Tc-wg* by pair-rule genes in *Tribolium* and discuss conserved and divergent functions of pair-rule genes in the regulation of segment polarity genes between *Drosophila* and *Tribolium*.

A model of regulation of *Tc-en* and *Tc-wg* by *Tribolium* pair-rule genes

A model of regulation of *Tc-en* and *Tc-wg* by *Tribolium* pair-rule genes is detailed in Fig 3.4*a*. *Tc-en* is activated where the secondary stripes of *Tc-eve* and *Tc-prd* overlap one

another (labeled *Tc-eve* a and *Tc-prd* a in the Fig 3.4*a*). *Tc-wg*, which is also activated by *Tc-prd* a, is repressed by *Tc-eve* a, restricting its expression to cells in even-numbered parasegments immediately anterior to stripes of *Tc-en* expression. Therefore, the primary pair-rule gene, *Tc-eve*, is required as a repressor of *Tc-wg* and, in addition to the secondary pair-rule gene, *Tc-prd*, as a coactivator of *Tc-en*, to generate the juxtaposed stripes of *Tc-wg* and *Tc-en* 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 overlapping secondary stripes of *Tc-run* and *Tc-eve* (*Tc-eve* b in Fig. 3.4*a*). *Tc-wg* is activated by the stronger stripe of *Tc-slp* (*Tc-slp* a in Fig. 3.4*a*), but repressed by *Tc-eve* b, restricting its expression to cells in even-numbered parasegments immediately anterior to the *Tc-en* stripes. Thus, the primary pair-rule genes, *Tc-eve* and *Tc-run*, act as coactivators of *Tc-en*, and *Tc-eve* acts as a repressor of *Tc-wg* to define the parasegmental boundary. Previously, we suggested that the primary gene circuit, composed of *Tc-eve*, *Tc-run* and *Tc-odd*, plays an important role to prepattern a two-segment wide region at the posterior end of the growth zone, whereas *Tc-prd* and *Tc-slp* are important to form odd- and even-numbered segments, respectively (2). Taken together with our previous results, our current model of the regulation of *Tc-en* and *Tc-wg* by *Tribolium* pair-rule genes suggests that the primary pair-rule genes, *Tc-eve* and *Tc-run*, are redeployed to regulate *Tc-en* and *Tc-wg* with *Tc-prd* and *Tc-slp*, after they function in double segment prepatterning. Furthermore, our model suggests that the redeployed primary pair-rule genes function with *Tc-prd* and *Tc-slp* to regulate the

expression of *Tc-en* and *Tc-wg* rather than acting through other secondary pair-rule genes as in *Drosophila* to define the parasegmental boundaries.

Conserved and divergent aspects in pair-rule gene regulation of segment polarity genes in *Drosophila* and *Tribolium*

In *Drosophila*, *en* and *wg* stripes are initiated by combinations of secondary pair-rule genes (summarized in Fig. 3.4*b*). For example, *en* in odd-numbered parasegments is activated by *prd*, while *wg* in even-numbered parasegments is activated by *prd* and *slp* (4, 13, 14). In addition, *slp* stripes in even-numbered parasegments repress the expression of *en* there, defining the anterior boundary of *en* expression at the parasegmental border (15, 16). To define the other parasegmental border, *en* is activated by *ftz* in even-numbered parasegments, while the adjacent *wg* in odd-numbered parasegments is activated by *opa* (3-6).

In addition to the secondary pair-rule genes that control *en* and *wg* directly, *Drosophila* primary pair-rule genes regulate *en* and *wg* indirectly through the secondary pair-rule genes (Fig. 3.4*b*). For example, repression of *en* by *slp* is restricted by *eve* in odd-numbered parasegments to define the anterior boundary of *en* at the parasegmental border (16, 17). *slp* is required to repress *en* and maintain *wg* expression in even-numbered parasegments (13). Consequently, *eve* plays important roles to regulate the expression of *en* and *wg* within odd- and even-numbered parasegments, respectively, by regulating the expression of *slp* (Fig. 3.4*b*). Furthermore, high concentrations of *eve* in odd-numbered parasegments repress *prd* to regulate the posterior border of *prd* expression (17, 18). *prd* is required to activate *en* in

odd-numbered parasegments. Therefore, *eve* plays an important role to regulate the posterior border of *en* expression in odd-numbered parasegments, by regulating the expression of *prd*. In contrast to the indirect regulation of *en* and *wg* by *eve*, it should be noted that secondary *run* stripes in even-numbered parasegments seem to repress *en* directly (19, 20). Interestingly, in *Tribolium*, *Tc-eve* a in odd-numbered parasegments seems to restrict the expression of *Tc-wg* within the even-numbered parasegments by directly repressing *Tc-wg* expression in the odd-numbered parasegments rather than through regulation of *Tc-slp* (Fig. 3.4*a*). Furthermore, *Tc-eve* a is also required as a coactivator to activate *Tc-en* in odd-numbered parasegments (Fig. 3.4*a*). Therefore, even though in both insects *prd* is required to activate *en* in odd-numbered parasegments and *wg* in even-numbered parasegments, different mechanisms to define the parasegmental boundary between *en* and *wg* stripes have evolved in the *Drosophila* and *Tribolium* lineages (compare Fig. 3.4*a* to 3.4*b*). Interestingly, however, it has been suggested that the combination of *eve* and *prd* specifies *en* stripes in odd-numbered parasegments in *Drosophila* (4, 21), and overexpression of *prd* resulted in posterior expansion of these *en* stripes (22). Therefore, it is possible that the overall mechanism to activate *en* in odd-numbered parasegments by *eve* and *prd* is conserved in *Drosophila* and *Tribolium*.

To define the other parasegmental boundary, between *en* in even-numbered parasegments and *wg* in odd-numbered parasegments, *Drosophila* and *Tribolium* also 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. Furthermore, secondary *eve* expression is important for *ftz*-dependent *en* activation in

even-numbered parasegments by repressing *odd*, which represses *en* (4, 17, 23, 24). 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 (25, and Fig. 3.4*b*). Therefore, in *Drosophila*, primary pair-rule genes *eve* and *run* indirectly regulate *en* in even-numbered parasegments by regulating secondary pair-rule genes (Fig. 3.4*b*). However, *Tc-ftz* and *Tc-opa* are not functional in *Tribolium* segmentation (2, 7). *Tc-slp*, instead of *Tc-opa*, activates *Tc-wg* in odd-numbered parasegments 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-eve* seems to repress *Tc-wg* expression in even-numbered parasegments without repression of *Tc-slp*. Therefore, in *Tribolium*, primary pair-rule genes *Tc-eve* and *Tc-run*, with the secondary pair-rule gene *Tc-slp*, regulate *Tc-en* in even-numbered parasegments and adjacent *Tc-wg* in odd-numbered parasegments without regulating known secondary pair-rule genes.

Previously, we determined that not all homologs of *Drosophila* pair-rule genes participate in *Tribolium* segmentation (2). The limits of this candidate gene approach raise the possibility of the presence of other pair-rule genes to explain the conserved segmental expression of *en* and *wg* at parasegmental boundaries (2). Indeed, this possibility still cannot be ruled out. However, our double and triple RNAi analysis of *Tribolium* pair-rule genes suggests that this apparently smaller repertoire of pair-rule genes defines parasegments by redeploying the primary pair-rule genes. 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 (1). Recently, 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 (26), 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 (27). 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 (11, 12). 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 represses *wg* and, in combination with a coactivator (*Tc-prd* or *Tc-run*), activates *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 (28). However, *eve* is expressed in segmental, not pair-rule, stripes in other arthropods (28, 29). Thus it is likely that the ancestral pattern was segmental in insects. In contrast, *prd* expression in pair-rule stripes is largely conserved in insects (10, 30-33). 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 ancestral insects, which relies on segmental stripes of *eve* and pair-rule stripes of *prd* (Fig. 3.7*a*, which is published as supporting information on the PNAS web site). In this model, *prd* activates *en* and *wg*, while *eve* represses *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, this model explains how the segmental stripes of both *prd* and *eve* in other arthropods might regulate the expression of *wg* and *en* (Fig. 3.7*b*). In this model, the segmental *prd* stripes extend more anterior than those of *eve*. 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 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 *Tribolium* and *Drosophila*, and perhaps other insects. Comparative analysis of pair-rule regulation of the segment polarity genes in basal insects and arthropods will provide the necessary test of these models.

Materials and Methods

Parental RNAi

Parental RNAi was performed as described (28). 900 ng/µl (*Tc-eve*), 500 ng/µl (*Tc-run*, *Tc-prd* and *Tc-slp*), or 350 ng/µl (*Tc-odd*) of dsRNA were injected into pupae to knock down gene(s).

Immunocytochemistry and whole-mount in situ hybridization

Immunocytochemistry was carried out as described in (12) with the mAbs 2B8 (anti-Eve) diluted to 1/20 or the 4D9 (anti-En) diluted to 1/5 (Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa). Whole-mount in situ hybridization was performed as in (11) with Digoxigenin-labeled RNA probes.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01-HD29594) (to S.J.B.).

Reference List

1. Peel, A. D., Chipman, A. D. & Akam, M. (2005) Nat Rev Genet 6, 905-16.
2. Choe, C. P., Miller, S. C. & Brown, S. J. (2006) Proc Natl Acad Sci U S A 103, 6560-4.
3. Benedyk, M. J., Mullen, J. R. & DiNardo, S. (1994) Genes Dev. 8, 105-117.
4. DiNardo, S. & O'Farrell, P. H. (1987) Genes Dev 1, 1212-25.
5. Howard, K. & Ingham, P. (1986) Cell 44, 949-57.
6. Ish-Horowicz, D., Pinchin, S. M., Ingham, P. W. & Gyurkovics, H. G. (1989) Cell 57, 223-32.
7. Stuart, J. J., Brown, S. J., Beeman, R. W. & Denell, R. E. (1991) Nature 350, 72-4.
8. Brown, S. J., Hilgenfeld, R. B. & Denell, R. E. (1994) Proc.Natl.Acad.Sci.U.S.A 91, 12922-12926.
9. Nagy, L. M. & Carroll, S. (1994) Nature 367, 460-3.
10. Choe, C. P. & Brown, S. J. (2006) Developmental Biology doi:10.1016/j.ydbio.2006.09.037.
11. Brown, S. J., Parrish, J. K., Beeman, R. W. & Denell, R. E. (1997) Mech.Dev. 61, 165-173.
12. Patel, N. H., Condron, B. G. & Zinn, K. (1994) Nature 367, 429-434.
13. Cadigan, K. M., Grossniklaus, U. & Gehring, W. J. (1994) Genes Dev. 8, 899-913.

14. Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A. A., Weir, M. & Goto, T. (1996) Development 122, 2697-707.

15. Cadigan, K. M., Grossniklaus, U. & Gehring, W. J. (1994) Proc.Natl.Acad.Sci.U.S.A 91, 6324-6328.

16. Jaynes, J. B. & Fujioka, M. (2004) Dev Biol 269, 609-22.

17. Fujioka, M., Jaynes, J. B. & Goto, T. (1995) Development 121, 4371-82.

18. Baumgartner, S. & Noll, M. (1990) Mech.Dev. 33, 1-18.

19. Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M. & Gergen, J. P. (1997) Mol Cell Biol 17, 5581-7.

20. Manoukian, A. S. & Krause, H. M. (1993) Development 118, 785-96.

21. Ingham, P. W., Baker, N. E. & Martinez-Arias, A. (1988) Nature 331, 73-75.

22. Morrissey, D., Askew, D., Raj, L. & Weir, M. (1991) Genes Dev. 5, 1684-1696.

23. Coulter, D. E. & Wieschaus, E. (1988) Genes Dev. 2, 1812-1823.

24. Manoukian, A. S. & Krause, H. M. (1992) Genes Dev 6, 1740-51.

25. Swantek, D. & Gergen, J. P. (2004) Development 131, 2281-90.

26. von Dassow, G., Meir, E., Munro, E. M. & Odell, G. M. (2000) Nature 406, 188-92.

27. Macdonald, P. M., Ingham, P. & Struhl, G. (1986) Cell 47, 721-34.

28. Liu, P. Z. & Kaufman, T. C. (2005) Development 132, 2081-2092.

29. Damen, W. G., Janssen, R. & Prpic, N. M. (2005) Evol.Dev. 7, 618-628.

30. Davis, G. K., Jaramillo, C. A. & Patel, N. H. (2001) Development 128, 3445-3458.

31. Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E. & Noll, M. (1986) Nature 321, 493-499.

32. Osborne, P. W. & Dearden, P. K. (2005) Dev Genes Evol 215, 499-508.

33. Davis, G. K., D'Alessio, J. A. & Patel, N. H. (2005) Dev Biol 285, 169-84.

34. Bucher, G., Scholten, J. & Klingler, M. (2002) Curr.Biol. 12, R85-R86.

Table 3.1 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 **-** abolishment 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

Figures

Figure 3.1 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 and other 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 *Tc-eve*^{RNAi} embryo, the expression of Tc-En (punctate, brown spots) is abolished except for the antennal stripe whereas *Tc-wg* (purple) 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 (arrows). *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 antennal stripes have not yet formed, but the mandibular and maxillary Tc-En (punctate, brown spots) and *Tc-wg* (purple) stripes form normally. Tc-En 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.

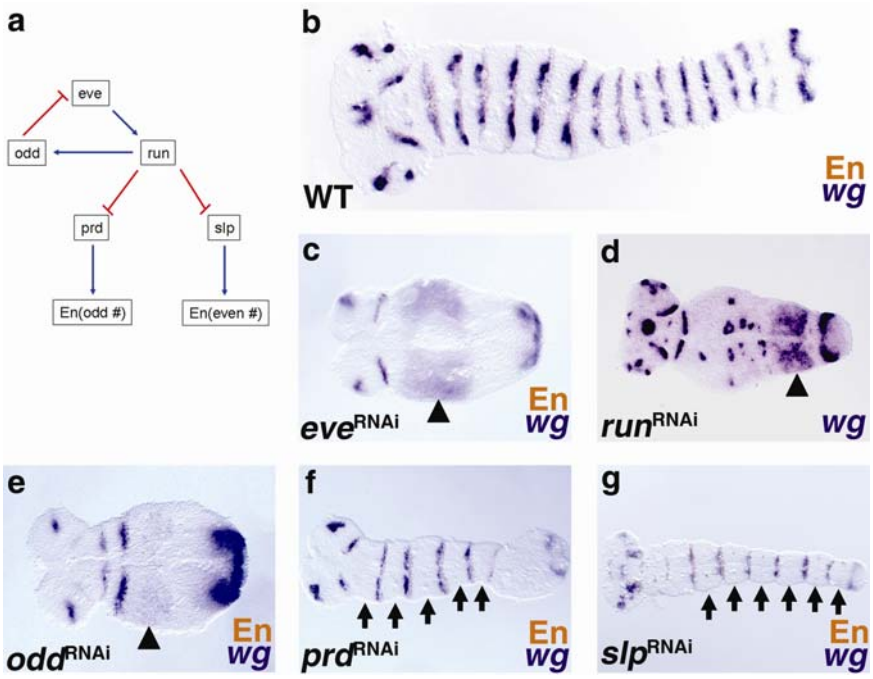

a
eve
odd
run
prd
slp
En(odd #)
En(even #)
b
WT
En
wg
c
eveRNAi
En
wg
d
runRNAi
wg
e
oddRNAi
En
wg
f
prdRNAi
En
wg
g
slpRNAi
En
wg

Figure 3.2 Analysis of genetic interactions affecting *Tc-en* and *Tc-wg* expression at the anterior boundary of odd-numbered parasegments.

In these ventral views, anterior is to the left. Double RNAi combinations are denoted by (*x*^*y*)^{RNAi}. (*a-d*) The young embryos shown here have not yet developed *Tc-en* and *Tc-wg* expression in the antennae. In the (*eve*^*slp*) double RNAi embryos, *Tc-en* (purple) is not expressed (*a*) whereas *Tc-wg* (purple) is expressed (*b*). *Tc-prd* (purple) is expressed broadly (*c*) whereas the expression of Tc-Eve (expected as punctate brown spots) is abolished by RNAi (*d*). (*e-h*) In the (*run*^*slp*) double RNAi embryos, *Tc-en* (purple) is expressed broadly (*e*) whereas *Tc-wg* (purple) expression in the trunk is not initiated (*f*). *Tc-prd* (purple) and Tc-Eve (punctate, brown spots) are expressed broadly (*g, h*) in these embryos.

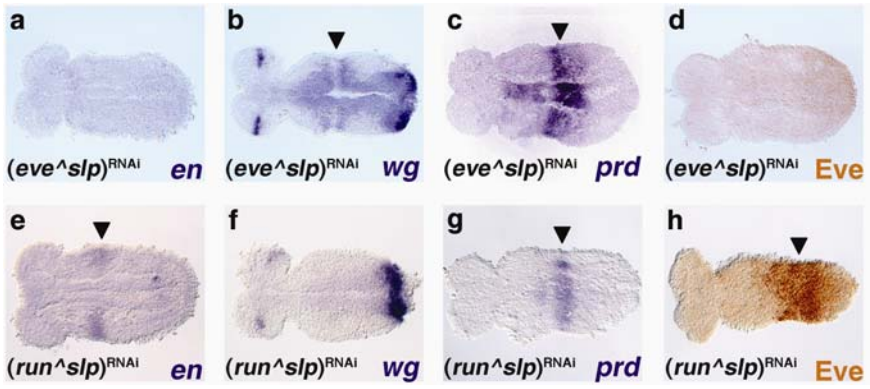


Figure 3.3 Analysis of genetic interactions affecting *Tc-en* and *Tc-wg* expression at the anterior boundary of even-numbered parasegments.

(*a-c*) In (*eve^prd*) double RNAi embryos, *Tc-wg* (purple) and *Tc-slp* (purple) are expressed broadly (*a, b*) whereas Tc-Eve (expected in punctate, brown spots) is not expressed (*c*). (*d-f*) In (*run ^prd*) RNAi embryos, *Tc-wg* (purple) is expressed weakly in a very narrow region (*d*), whereas *Tc-slp* (purple) (*e*) and Tc-Eve (punctate, brown spots) (*f*) are expressed ectopically. (*g-i*) In *Tc-odd*^{RNAi} embryos, *Tc-en* (purple) is expressed normally in mandibular and maxillary segments and in a broad central region (*g*). Tc-Eve (punctate, brown spots) (*h*) and *Tc-run* (purple) (*i*) are also expressed ectopically in the remaining tissue including the presumptive growth zone. (*j-l*) In *Tc-run*, *Tc-prd*, *Tc-slp* triple RNAi embryos, *Tc-en* (purple) and *Tc-run* (purple) are not expressed (*j*, *l*), whereas Tc-Eve (punctate, brown spots) is expressed ectopically (*k*).

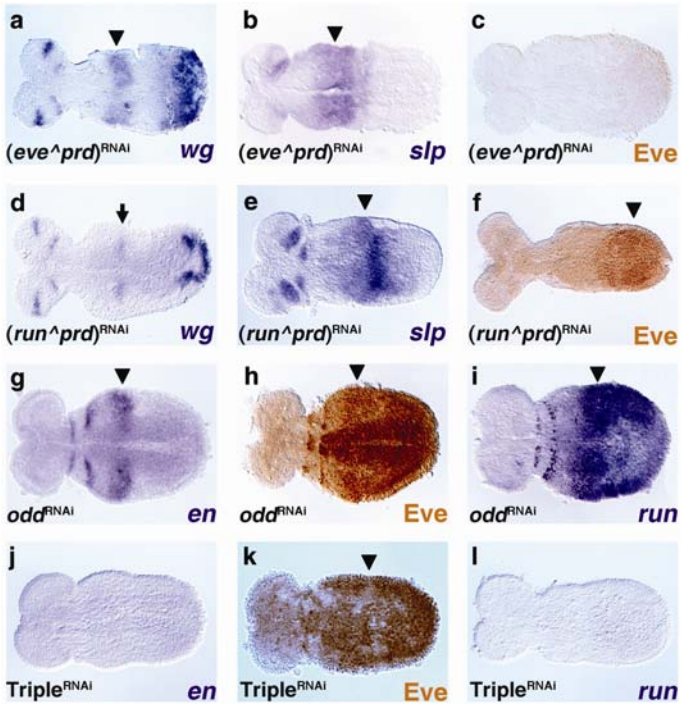
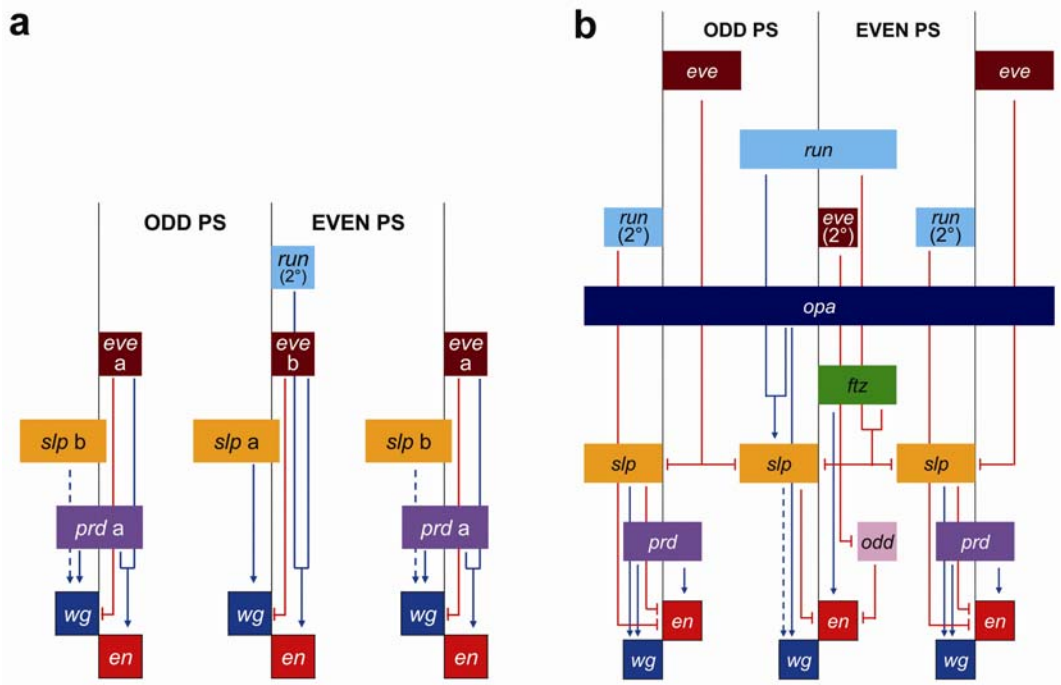


Figure 3.4 Regulation of *en* and *wg* by pair-rule genes in *Tribolium* and *Drosophila*.

(*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. Direct activation and repression are in blue and red lines, respectively, and maintenance interactions are denoted by broken blue lines.


a
ODD PS
EVEN PS
run (2°)
eve a
eve b
eve a
slp b
slp a
slp b
prd a
prd a
wg
wg
wg
en
en
en
b
ODD PS
EVEN PS
eve
eve
run
run (2°)
eve (2°)
run (2°)
opa
ftz
slp
slp
slp
prd
odd
prd
en
en
en
wg
wg
wg

Supporting Figures

Figure 3.5 Expression of *Tc-run* and *Tc-slp* in double RNAi embryos of *Tc-eve* or *Tc-run* in combination with *Tc-slp*.

(*a*, *b*) In these (*eve^slp*) double RNAi embryos, the expression of *Tc-run* (purple) and *Tc-slp* (purple) is abolished. (*c*, *d*) In these (*run^slp*) double RNAi embryos, *Tc-run* (purple) and *Tc-slp* (purple) are not expressed.

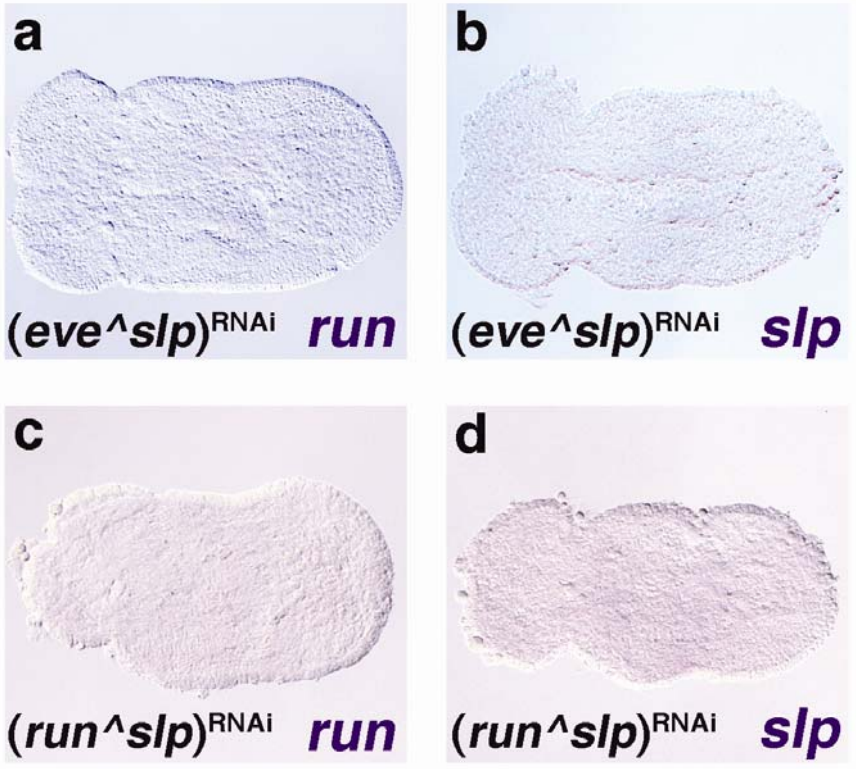


Figure 3.6 Expression of pair-rule genes in double RNAi embryos of *Tc-eve* and *Tc-prd*, *Tc-run* and *Tc-prd*, in *Tc-odd*^{RNAi} embryos, and in triple RNAi embryos of *Tc-run*, *Tc-prd* and *Tc-slp*.

(*a*, *b*) In these (*eve^prd*) double RNAi embryos *Tc-run* (purple) and *Tc-prd* (purple) are not expressed. (*c*, *d*) In these (*run^prd*) double RNAi embryos of *Tc-run* and *Tc-prd*, expression of *Tc-run* (purple) and *Tc-prd* (purple) is abolished. (*e*, *f*) In these triple RNAi embryos of *Tc-run*, *Tc-prd* and *Tc-slp*, *Tc-prd* (purple) and *Tc-slp* (purple) were successfully knocked down.

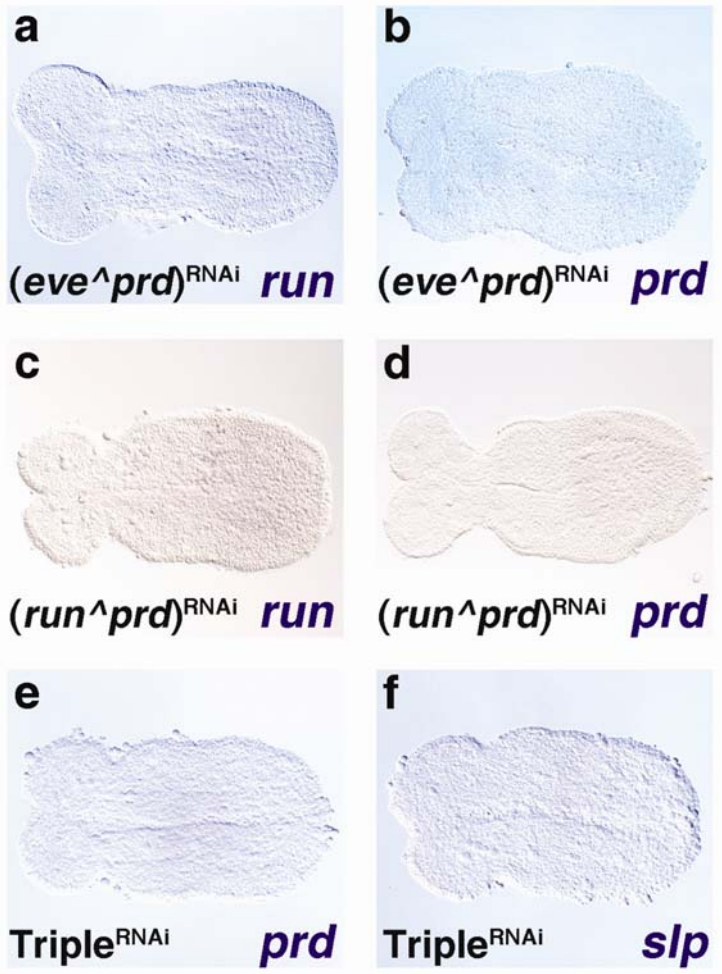
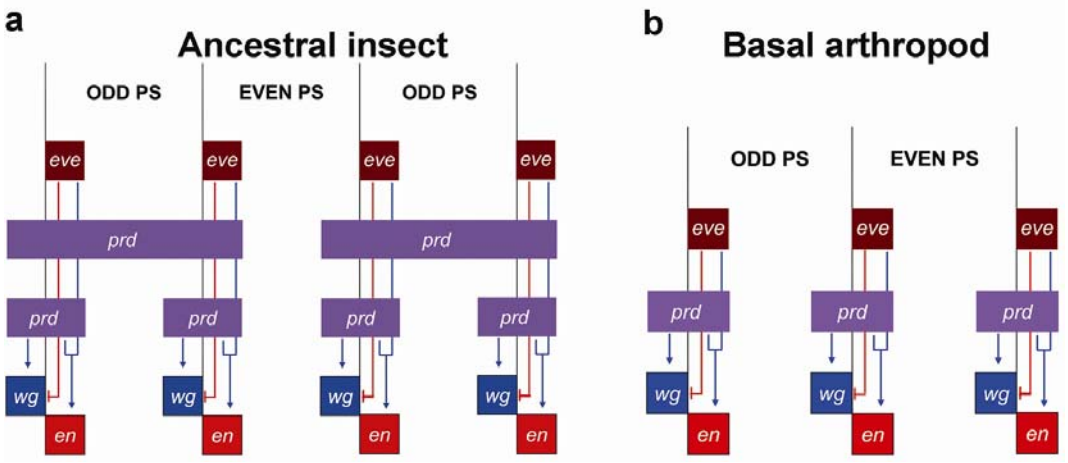


Figure 3.7 Pair-rule regulation of *en* and *wg* in ancestral insects and arthropods

(*a*) 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* prepattern units that are two-segment wide and then are resolved into segmental stripes. The segmental stripes activate *en* and *wg* at each parasegmental boundary while segmental stripes of *eve* are coincident with *en* stripes to suppress prd-dependent *wg* activation. (*b*) Regulation of *en* and *wg* by segmental stripes of *eve* and *prd* in basally branching arthropods. In this model, each stripe of *eve* is coincident with an *en* stripe while segmental stripes of *prd* are overlapped both *en* and *wg* stripes. *prd* is required to activate *en* and *wg* there whereas *eve* suppresses the activation of *wg* by *prd* in *en* expressing cells.



References

Anderson, P. (1995). Mutagenesis. *Methods Cell Biol* **48**, 31-58.

Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M. and Gergen, J. P. (1997). Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol Cell Biol* **17**, 5581-7.

Baumgartner, S. and Noll, M. (1990). Network of interactions among pair-rule genes regulating *paired* expression during primordial segmentation of *Drosophila*. *Mech.Dev.* **33**, 1-18.

Benedyk, M. J., Mullen, J. R. and DiNardo, S. (1994). *odd-paired*: a zinc finger pair-rule protein required for the timely activation of *engrailed* and *wingless* in *Drosophila* embryos. *Genes Dev.* **8**, 105-117.

Bennett, R. L., Brown, S. J. and Denell, R. E. (1999). Molecular and genetic analysis of the *Tribolium Ultrabithorax* ortholog, *Ultrathorax*. *Dev.Genes Evol.* **209**, 608-619.

Bertuccioli, C., Fasano, L., Jun, S., Wang, S., Sheng, G. and Desplan, C. (1996). In vivo requirement for the paired domain and homeodomain of the *paired* segmentation gene product. *Development* **122**, 2673-2685.

Brown, S., Holtzman, S., Kaufman, T. and Denell, R. (1999a). Characterization of the *Tribolium Deformed* ortholog and its ability to directly regulate *Deformed* target genes in the rescue of a *Drosophila Deformed* null mutant. *Dev.Genes Evol.* **209**, 389-398.

Brown, S. J. and Denell, R. E. (1996). Segmentation and dorsoventral patterning in *Tribolium*. *Sem. Cell Dev. Biol.* **7**, 553-560.

Brown, S. J., Hilgenfeld, R. B. and Denell, R. E. (1994a). The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc.Natl.Acad.Sci.U.S.A* **91**, 12922-12926.

Brown, S. J., Mahaffey, J. P., Lorenzen, M. D., Denell, R. E. and Mahaffey, J. W. (1999b). Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evol.Dev.* **1**, 11-15.

Brown, S. J., Parrish, J. K., Beeman, R. W. and Denell, R. E. (1997). Molecular characterization and embryonic expression of the *even-skipped* ortholog of *Tribolium castaneum*. *Mech.Dev.* **61**, 165-173.

Brown, S. J., Patel, N. H. and Denell, R. E. (1994b). Embryonic expression of the single *Tribolium engrailed* homolog. *Dev Genet* **15**, 7-18.

Bucher, G. and Klingler, M. (2004). Divergent segmentation mechanism in the short germ insect *Tribolium* revealed by *giant* expression and function. *Development* **131**, 1729-1740.

Bucher, G., Scholten, J. and Klingler, M. (2002). Parental RNAi in *Tribolium* (Coleoptera). *Curr.Biol.* **12**, R85-R86.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994a). Functional redundancy: the respective roles of the two *sloppy paired* genes in *Drosophila* segmentation. *Proc.Natl.Acad.Sci.U.S.A* **91**, 6324-6328.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994b). Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments. *Genes Dev.* **8**, 899-913.

Cerny, A. C., Bucher, G., Schroder, R. and Klingler, M. (2005). Breakdown of abdominal patterning in the *Tribolium Kruppel* mutant *jaws*. *Development* **132**, 5353-5363.

Chipman, A. D., Arthur, W. and Akam, M. (2004). A double segment periodicity underlies segment generation in centipede development. *Curr.Biol.* **14**, 1250-1255.

Choe, C. P. and Brown, S. J. (2006). 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*. *Dev Biol* doi:10.1016/j.ydbio.2006.09.037

Choe, C. P., Miller, S. C. and Brown, S. J. (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. *Proc Natl Acad Sci U S A* **103**, 6560-4.

Copf, T., Schroder, R. and Averof, M. (2004). Ancestral role of *caudal* genes in axis elongation and segmentation. *Proc Natl Acad Sci U S A* **101**, 17711-5.

Coulter, D. E. and Wieschaus, E. (1988). Gene activities and segmental patterning in *Drosophila*: analysis of *odd-skipped* and pair-rule double mutants. *Genes Dev.* **2**, 1812-1823.

Curtis, C. D., Brisson, J. A., DeCamillis, M. A., Shippy, T. D., Brown, S. J. and Denell, R. E. (2001). Molecular characterization of *Cephalothorax*, the Tribolium ortholog of *Sex combs reduced*. *Genesis*. **30**, 12-20.

Damen, W. G., Hausdorf, M., Seyfarth, E. A. and Tautz, D. (1998). A conserved mode of head segmentation in arthropods revealed by the expression pattern of Hox genes in a spider. *Proc.Natl.Acad.Sci.U.S.A* **95**, 10665-10670.

Damen, W. G., Janssen, R. and Prpic, N. M. (2005). Pair rule gene orthologs in spider segmentation. *Evol.Dev.* **7**, 618-628.

Davis, G. K., Jaramillo, C. A. and Patel, N. H. (2001). Pax group III genes and the evolution of insect pair-rule patterning. *Development* **128**, 3445-3458.

Davis, G. K. and Patel, N. H. (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annu.Rev.Entomol.* **47**, 669-699.

Davis, G. K. and Patel, N. H. (2003). Playing by pair-rules? *Bioessays* **25**, 425-9.

Davis, G. K., D'Alessio, J. A. and Patel, N. H. (2005). Pax3/7 genes reveal conservation and divergence in the arthropod segmentation hierarchy. *Dev Biol* **285**, 169-84.

Dawes, R., Dawson, I., Falciani, F., Tear, G. and Akam, M. (1994). *Dax*, a locust Hox gene related to *fushi-tarazu* but showing no pair-rule expression. *Development* **120**, 1561-1572.

Dearden, P. K., Donly, C. and Grbic, M. (2002). Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite *Tetranychus urticae*. *Development* **129**, 5461-5472.

DiNardo, S. and O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by pair-rule genes. *Genes Dev* **1**, 1212-25.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation gene *paired* and the Drosophila PRD gene set as part of a gene network. *Cell* **47**, 735-746.

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B. (1999). Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* **126**, 2527-38.

Fujioka, M., Jaynes, J. B. and Goto, T. (1995). Early *even-skipped* stripes act as morphogenetic gradients at the single cell level to establish *engrailed* expression. *Development* **121**, 4371-82.

Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A. A., Weir, M. and Goto, T. (1996). *Drosophila* Paired regulates late *even-skipped* expression through a composite binding site for the paired domain and the homeodomain. *Development* **122**, 2697-707.

Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M. and Engels, W. R. (1993). Type I repressors of P element mobility. *Genetics* **135**, 81-95.

Grossniklaus, U., Cadigan, K. M. and Gehring, W. J. (1994). Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head. *Development* **120**, 3155-3171.

Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1992). The *Drosophila sloppy paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. *Genes Dev.* **6**, 1030-1051.

Howard, K. and Ingham, P. (1986). Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy*, and *engrailed* in the *Drosophila* blastoderm. *Cell* **44**, 949-57.

Hulskamp, M. and Tautz, D. (1991). Gap genes and gradients--the logic behind the gaps. *Bioessays* **13**, 261-8.

Ingham, P. and Gergen, P. (1988). Interactions between the pair-rule genes *runt*, *hairy*, *even-skipped* and *fushi tarazu* and the establishment of periodic pattern in the *Drosophila* embryo. *Development* **104** Suppl., 51-60.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature* **335**, 25-34.

Ingham, P. W., Baker, N. E. and Martinez-Arias, A. (1988). Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped*. *Nature* **331**, 73-75.

Ingham, P. W. and Martinez-Arias, A. (1986). The correct activation of Antennapedia and bithorax complex genes requires the *fushi tarazu* gene. *Nature* **324**, 592-597.

Irvine, K. D. and Wieschaus, E. (1994). Cell intercalation during *Drosophila* germband extension and its regulation by pair-rule segmentation genes. *Development* **120**, 827-41.

Ish-Horowicz, D., Pinchin, S. M., Ingham, P. W. and Gyurkovics, H. G. (1989). Autocatalytic *ftz* activation and metameric instability induced by ectopic *ftz* expression. *Cell* **57**, 223-32.

Jaynes, J. B. and Fujioka, M. (2004). Drawing lines in the sand: *even skipped* et al. and parasegment boundaries. *Dev Biol* **269**, 609-22.

Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E. and Noll, M. (1986). Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. *Nature* **321**, 493-499.

Lawrence, P. A. (1992). The Making of a Fly: the Genetics of Animal Design. Oxford, UK: Blackwell Scientific.

Liu, P. Z. and Kaufman, T. C. (2004). *hunchback* is required for suppression of abdominal identity, and for proper germband growth and segmentation in the intermediate germband insect *Oncopeltus fasciatus*. *Development* **131**, 1515-27.

Liu, P. Z. and Kaufman, T. C. (2005). *even-skipped* is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect. *Development* **132**, 2081-2092.

Lynch, J. A., Brent, A. E., Leaf, D. S., Pultz, M. A. and Desplan, C. (2006). Localized maternal *orthodenticle* patterns anterior and posterior in the long germ wasp *Nasonia*. *Nature* **439**, 728-32.

Macdonald, P. M. and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**, 537-45.

Macdonald, P. M., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of *Drosophila* containing a homeo box. *Cell* **47**, 721-34.

Maderspacher, F., Bucher, G. and Klingler, M. (1998). Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. *Dev.Genes Evol.* **208**, 558-568.

Manoukian, A. S. and Krause, H. M. (1992). Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos. *Genes Dev* **6**, 1740-51.

Manoukian, A. S. and Krause, H. M. (1993). Control of segmental asymmetry in *Drosophila* embryos. *Development* **118**, 785-96.

Martizez Arias, A., Baker, N. E. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 157-70.

Mito, T., Sarashina, I., Zhang, H., Iwahashi, A., Okamoto, H., Miyawaki, K., Shinmyo, Y., Ohuchi, H. and Noji, S. (2005). Non-canonical functions of *hunchback* in segment patterning of the intermediate germ cricket *Gryllus bimaculatus*. *Development* **132**, 2069-2079.

Mohler, J. and Wieschaus, E. F. (1985). *Bicaudal* mutations of *Drosophila* melanogaster: alteration of blastoderm cell fate. *Cold Spring Harb Symp Quant Biol* **50**, 105-11.

Morrissey, D., Askew, D., Raj, L. and Weir, M. (1991). Functional dissection of the *paired* segmentation gene in *Drosophila* embryos. *Genes Dev.* **5**, 1684-1696.

Nagy, L. M. and Carroll, S. (1994). Conservation of *wingless* patterning functions in the short-germ embryos of *Tribolium castaneum*. *Nature* **367**, 460-3.

Nasiadka, A., Dietrich, B. H. and Krause, H. M. (2002). Anterior–posterior patterning in the *Drosophila* embryo. *Adv. Dev. Biol. Biochem.* **12**, 155-204.

Niessing, D., Rivera-Pomar, R., La Rosee, A., Hader, T., Schock, F., Purnell, B. A. and Jackle, H. (1997). A cascade of transcriptional control leading to axis determination in *Drosophila*. *J.Cell Physiol* **173**, 162-167.

Nulsen, C. and Nagy, L. M. (1999). The role of *wingless* in the development of multibranched crustacean limbs. *Dev.Genes Evol.* **209**, 340-348.

Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801.

Osborne, P. W. and Dearden, P. K. (2005). Expression of Pax group III genes in the honeybee (*Apis mellifera*). *Dev.Genes Evol.* **215**, 499-508.

Patel, N. H., Ball, E. E. and Goodman, C. S. (1992). Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature* **357**, 339-342.

Patel, N. H., Condron, B. G. and Zinn, K. (1994). Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. *Nature* **367**, 429-434.

Patel, N. H., Hayward, D. C., Lall, S., Pirkl, N. R., DiPietro, D. and Ball, E. E. (2001). Grasshopper *hunchback* expression reveals conserved and novel aspects of axis formation and segmentation. *Development* **128**, 3459-3472.

Peel, A. D., Chipman, A. D. and Akam, M. (2005). Arthropod segmentation: beyond the *Drosophila* paradigm. *Nat Rev Genet* **6**, 905-16.

Pourquie, O. (2001). Vertebrate somitogenesis. *Annu Rev Cell Dev Biol* **17**, 311-50.

Pourquie, O. (2003). The segmentation clock: converting embryonic time into spatial pattern. *Science* **301**, 328-330.

Saulier-Le Drean, B., Nasiadka, A., Dong, J. and Krause, H. M. (1998). Dynamic changes in the functions of Odd-skipped during early *Drosophila* embryogenesis. *Development* **125**, 4851-61.

Schoppmeier, M. and Damen, W. G. (2005). Expression of Pax group III genes suggests a single-segmental periodicity for opisthosomal segment patterning in the spider *Cupiennius salei*. *Evol.Dev.* **7**, 160-169.

Schroder, R. (2003). The genes *orthodenticle* and *hunchback* substitute for *bicoid* in the beetle *Tribolium*. *Nature* **422**, 621-5.

Schulz, C., Schroder, R., Hausdorf, B., Wolff, C. and Tautz, D. (1998). A *caudal* homologue in the short germ band beetle *Tribolium* shows similarities to both, the *Drosophila* and the vertebrate *caudal* expression patterns. *Dev Genes Evol* **208**, 283-9.

Sommer, R. J. and Tautz, D. (1993). Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* **361**, 448-450.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-19.

Stauber, M., Jackle, H. and Schmidt-Ott, U. (1999). The anterior determinant *bicoid* of *Drosophila* is a derived Hox class 3 gene. *Proc Natl Acad Sci U S A* **96**, 3786-9.

Stollewerk, A., Schoppmeier, M. and Damen, W. G. (2003). Involvement of *Notch* and *Delta* genes in spider segmentation. *Nature* **423**, 863-865.

Stuart, J. J., Brown, S. J., Beeman, R. W. and Denell, R. E. (1991). A deficiency of the homeotic complex of the beetle *Tribolium*. *Nature* **350**, 72-4.

Sulston, I. A. and Anderson, K. V. (1996). Embryonic patterning mutants of *Tribolium castaneum*. *Development* **122**, 805-14.

Swantek, D. and Gergen, J. P. (2004). Ftz modulates Runt-dependent activation and repression of segment-polarity gene transcription. *Development* 131, 2281-90.

Tautz, D. (2004). Segmentation. *Dev.Cell* **7**, 301-312.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

von Dassow, G., Meir, E., Munro, E. M. and Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature* **406**, 188-92.

Wolff, C., Schroder, R., Schulz, C., Tautz, D. and Klingler, M. (1998). Regulation of the *Tribolium* homologues of *caudal* and *hunchback* in *Drosophila*: evidence for maternal gradient systems in a short germ embryo. *Development* **125**, 3645-54.

Wolff, C., Sommer, R., Schroder, R., Glaser, G. and Tautz, D. (1995). Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*. *Development* **121**, 4227-36.